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# The Effect of Aerobic Exercise Training on Cerebrovascular HSP70, HSP90, INOS and ENOS Expression in Type 1 Diabetes

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Graduate Program in Kinesiology  
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science  
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**THE EFFECT OF AEROBIC EXERCISE TRAINING ON  
CEREBROVASCULAR HSP70, HSP90, INOS AND ENOS  
EXPRESSION IN TYPE 1 DIABETES**

Cerebrovascular HSP & NOS in Exercise Training and T1DM

Thesis Format: Integrated Article

By

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Graduate Program in Kinesiology

A thesis submitted in partial fulfillment of  
the requirements for the degree of  
Master of Science

School of Graduate and Postdoctoral Studies  
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## **ABSTRACT**

The purpose of this study was to determine the effect of exercise training alone and in a model of Type 1 Diabetes Mellitus (T1DM), on Heat Shock Protein (HSP) and Nitric Oxide Synthase (NOS) expression in entorhinal adjacent large and small cerebral vessels. Thirty-two rats were randomly allocated to four groups: control sedentary (C), control exercised (CX), diabetic sedentary (D) and diabetic exercised (DX). Exercise training incorporated 5 days/week on a motorized treadmill (27m/min; 6 degree incline; 1 hour) for 10 weeks. Exercise trained groups had significantly greater Hsp70 expression than their respective non-trained groups ( $p<0.05$ ) and this response was not blunted in T1DM animals. The inducible NOS (iNOS) expression was greater in diabetic sedentary when compared to all other groups ( $p<0.001$ ). Co-localization of protein with smooth muscle cells illustrates that all HSP and NOS signal content is localized to the smooth muscle area (SMA).

Keywords: cerebral vessels, exercise, type 1 diabetes mellitus, Heat shock protein 70, inducible nitric oxide synthase

## **CO-AUTHORSHIP**

Dr. Earl Noble was involved in project design, interpretation of the results and thesis revisions.

## EPIGRAPH

We just keep playing that way.

I think it's the way the game is supposed to be played;

Either if you win or lose, you have to keep playing hard till the last out.

*-David 'Big Papi' Ortiz #34 Red Sox Vs. Yankees-Post Game 4 (8/17/2004)*

## DEDICATION

*To the giants in this life...*

The pillars of nature and nurture, HR Dey and S Dey  
The younger, the braver S Dey  
Et ma raison pour continuer, JW Fraser

*If I have seen further it is by standing on your shoulders*  
Adapted from Isaac Newton, 1675

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Team DI-CVD: Matt McDonald, Ken Grisé, T. Dylan Olver, through our tenacious efforts to investigate plight of the ‘betes’ you have not only taught me the beautiful métier underlying a collaborative network but to a greater extent, the enlightening camaraderie that has made the labor priceless. To you three, it has been my pleasure.

Thank you to the Exercise Biochemistry family Michael Murray, Mao Jiang, Hana Kowalchuk, Guangyu Robbie Li, John Trevithick and of course ‘the’ Tomasz Dzialoszynski, for a fellowship that will always make me smile out loud in the years to come.

To the magnitude that is: my parents, who have been models of integrity and unconditional love; my brother, for taking a mere genetic bond to great heights; the Frasers for the limitless warmth; and my friends- who have all collectively with forbearance gotten me this far. And J.W.F..... For making my reality a dream

*~Il faut cultiver notre jardin-Candide, Voltaire~*

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## **LIST OF ABBREVIATIONS**

$\beta$ cell	$\beta$ cell of the pancreatic islets of Langerhans
CBF	Cerebral Blood Flow
CNS	Central Nervous System
C	Control Sedentary Animal Group
CX	Control Exercise Trained Animal Group
CVD	Cardiovascular disease
D	Animals in Sedentary Diabetic Group
DAPI	4', 6-diamidino-2-phenylindole
dH <sub>2</sub> O	Distilled water
ddH <sub>2</sub> O	Double distilled water
DM	Diabetes mellitus
DN	Diabetic neuropathy
DX	Animals in Exercise Trained Diabetic Group
eNOS	Endothelial nitric oxide synthase
EX	Exercise
HSE	Heat shock element
HSF1	Heat shock transcription factor 1
Hsc70	Constitutive heat shock protein
Hsp70/72	70/72 kDa inducible heat shock protein
Hsp90	90 kDa constitutive heat shock protein
IP	Intraperitoneal
kD	Kilodalton

MAP	Mean arterial pressure
MCAO	Middle cerebral artery occlusion
mmHg	Millimeters mercury
NO	Nitric oxide
OCT	Optimal Cutting Temperature mounting medium
PBS	Phosphate-buffered saline
ROS	Reactive oxygen species
SMA	Smooth Muscle Area
STZ	Streptozotocin
T1DM	Type 1 Diabetes Mellitus
VSMC	Vascular Smooth Muscle Cells
VO <sub>2</sub> max	Maximal volume of oxygen uptake

## CHAPTER 1

### 1.1 Introduction

Exercise is a physiological stressor that partially protects against a multitude of biological stressors which impose deleterious consequences on cellular and tissue homeostasis. Hence, exercise interventions are becoming increasingly important as they have been illustrated to function as a critical preventative and potential therapeutic tool under a variety of conditions. Indeed, aerobic exercise training is neuroprotective in an assembly of diseases that include but are not limited to brain ischemia tolerance in the event of ischemic stroke.

Strokes are the second leading cause of death worldwide. According to the American Heart Association, ischemic stroke accounts for 87% of the incidents while hemorrhagic and transient ischemic attacks (TIA) account for the remaining 13% (36, 62). Although exercise may limit the likelihood of and/or damage caused by stroke the exact mechanism(s) as to how exercise exerts its benefits is not well understood (14, 16, 17). However, the biological networks that elicit exercise-induced protection are complex. However the role of chaperone proteins in cellular stress response has been linked to stroke injury protection (42, 56).

Exercise is a physiological stimulus that elicits a systemic stress response on a multitude of molecular regulators (4, 18). A highly studied family of chaperone proteins known as heat shock proteins (HSPs) encompass inducible members that are activated by exercise stress such as the 70-kDA HSP (Hsp70) (28). Minor episodes of stress are capable of eliciting this heat shock response (HSR) and thereby conferring protection in the event of a major stressful event. In this regard, exercise pre-conditioning has been

found to stimulate the HSR and induce Hsp70, which in turn prevents and rescues extensive protein misfolding thereby building systemic and potentially brain ischemia tolerance (81). Subsequent research in experimental T1DM models hinted at overexpression-associated neuroprotection protecting and/or curbing the severity of focal and global cerebral ischemia (68). Exercise pre-conditioning increases brain ischemia tolerance by inducing the HSR which in return helps improve recovery in diseases that are susceptible to protein misfolding and inflammation .

Ischemic stroke prevalence has increased globally in conjunction with the growing Type 1 Diabetes Mellitus (T1DM) epidemic. Epidemiological studies from the American Stroke Association indicate a significantly high risk factor for ischemic stroke in patients with T1DM regardless of age and the process of aging itself, independently increases the prevalence of ischemic stroke (23, 62). T1DM related hyperglycemia may cause endothelial dysfunction and accelerated atherosclerosis, notable precursors that lead to ischemic stroke. Particularly, the inflammatory consequences and the ensuing endothelial dysfunction exhibited in T1DM etiology have been associated with metabolic shifts in nitric oxide (NO) and nitric oxide synthase (NOS) regulation (22, 63). NO balance is critical for cellular function and is maintained by physiological production of NO by eNOS but can reach toxic levels due to the inducible NOS (iNOS) (22, 28, 45). In the T1DM profile, there is a pattern of decreased NO bioactivity with decreased eNOS content and in some cases, this observation is concurrent with an overproduction of pro-inflammatory iNOS (21, 32). In addition to the general protection against protein misfolding conferred by Hsp70, this protein was recently shown to elicit an anti-inflammatory effect through the inhibition of iNOS (1, 35). This chapter will provide an



overview of the neuroprotective role of exercise pre-conditioning in cerebral health and disease, with particular attention to heat shock protein regulation as a potential mechanism conferring this protection. The overview will incorporate a perspective on aerobic exercise training and the changes associated with T1DM on brain health with emphasis on the Hsp70, Hsp90, iNOS and eNOS and how these respective protein expressions are characterized in diabetes alone, and with exercise training interventions.

## **1.2 T1DM and the Progression of Cardiac and Vascular Consequences**

According to the American Diabetes Association (ADA), T1DM although not as prevalent as T2DM, remains a growing global concern (7). T1DM is an autoimmune disorder caused by the immune mediated destruction of pancreatic  $\beta$  cells, resulting in an significant decline in the production of insulin (58). Individuals with T1DM undergo exogenous insulin therapy, however, long-term glycemic complications persist and fluctuations between hyperglycemic and hypoglycemic states impose distress to daily life. The Framingham study report from 1979 on diabetes and cardiovascular implications established that patients with diabetes had a high prevalence of peripheral artery disease, coronary disease, and strokes (33, 77). Diabetes itself is associated with impaired tissue defense mechanisms that render the body vulnerable to various stressors. One such stressor is oxidative stress, which potentially plays a significant role in diabetes related complications (37). Hyperglycemia and hypertension are two atherogenic factors underlying T1DM macro- and micro- vascular complications that evolve to become primary contributors to injurious cardiovascular events, stroke, or peripheral vascular disease (52, 58). These atherogenic factors contribute to the development of oxidative stress, an imbalance in nitric oxide bioavailability and the production of reactive oxygen

species (ROS) that collectively contribute to endothelial dysfunction (ED) implicated in T1DM induced stroke and cardiovascular complications (52, 58, 63).

### **1.3 Histological Perspectives on Cerebrovasculature**

Blood vessels form an intricate network throughout the central and peripheral system. The innermost layer of blood vessel, the tunica intima or endothelium, is the immediate contact to the circulation and behaves as a selective barrier to constituents in the blood. In the case of larger blood vessels, the endothelium is surrounded by a tunica media layer of vascular smooth muscle cells (VSMC) that are responsible for vasodilation and vasoconstriction (71, 74). Amongst other methods, the endothelium governs vasodilation through nitric oxide synthesis and vasoconstriction through the synthesis of endothelin (74). In peripheral circulation, blood vessels supply tissues with nutrients upon cellular demand. Exercise creates an environment of high metabolic demand and initiates physiological changes that include fluxes in oxidative stress, sheer stress and changes in perfusion pressure that initiate vascular responses in order to divert nutrients to the site of working muscles (29). The central nervous system circulation varies from that of peripheral, because the brain requires a more constant blood flow. Though exercise-induced vascular responses have been investigated in skeletal muscle and myocardial vasculature, very little is known of the mechanisms or components underlying molecular changes associated with exercise stress in cerebral vessels. In T1DM, progressive metabolic instability leads to structural changes in the vessels and the consequences lead to events of ischemic stroke (58). An aspect of therapeutic stroke research is targeted towards post-stroke injury and attenuating the degree of injury. It is illustrated that stroke induced inflammatory reactions in the vessel, is on such mechanism

underlying prolonged post-stroke injury and evidenced by Kusaka et al. , who illustrated underlying causes of prolonged post-stroke injury to a decreased reactivity of cerebral arterioles during reperfusion (37).

The mammalian brain is sensitive to oxidative stress and this is evident in the aged brain. With aging, significant and progressive increases in the level of oxidative stress have been linked to the development of various neurodegenerative conditions such as Alzheimer's disease. Studies with a neurodegenerative Alzheimer's model have indicated that the hippocampus and adjacent areas, experience vast changes with aging and that they are particularly sensitive to stress due to a high regional concentration of glucocorticoid receptors (3, 20). In recent years Brown et.al established neuronal expression of heat shock protein in rat brain, particular to the hippocampus and adjacent the entorhinal cortex matter (9). A recent study has demonstrated a link between the aged brain that exhibits the Alzheimer's phenotype and that of a T1DM brain and have postulated a potential glucoregulatory mechanism underlying the progression of Alzheimer's etiology (6). Similar investigations to the aforementioned neurodegenerative models have illustrated neuronal HSR in areas of the mammalian cerebrum that were confined to the spinal cord, hippocampus and entorhinal cortex. The latter two locales in the rat mid-brain have been the most susceptible to disease induced neuronal loss including ischemic stroke (19, 60). Though tissue specific heat shock has been identified in cerebral tissue, very little is known of the cerebrovascular response to T1DM.

#### **1.4 Physiological Implications of T1DM and Stroke**

There is growing evidence of end-organ injury in the central nervous system that has been attributed to hyperglycemia mediated metabolic and endocrine disturbances (7). These disruptions lead to structural shifts in vascular morphology and functional shifts in cerebral blood flow (CBF), which collectively manifest to progressive atherosclerosis in diabetes and may induce stroke (65, 75). The incidence of ischemic stroke is two to six folds higher in patients with diabetes when compared to those without diabetes (77).

Diabetes affects the cerebrovascular circulation by increasing the risk of intracranial and extra cranial atherosclerosis (7, 46). In diabetic cerebral microvasculature, structural irregularities include decreased capillary density and thickening of capillary basement membranes (5, 34). Preliminary data asserts microvasculature injury appears to manifest prior to the onset of clinical symptoms, however little else is characterized in the microvasculature to bolster these assertions (7).

More so than other organs, the brain requires continuous circulating glucose and cerebral health is tightly managed by glucoregulatory control. The brain requires 1/5 of the available oxygen uptake at rest for survival and CBF is tightly regulated to ensure sufficient nutrient and oxygen delivery (61). In a normotensive state, CBF is relatively constant and maintained at arterial blood pressure of 60 to 160mmHg in order to circumvent fluctuations in perfusion, that could lead to a state of hypoxia or potentially increase in intracranial pressure (55, 61). Cerebral autoregulation, a marked feature of the developed mammalian brain, refers to the capacity of cerebral vessels to compensate for and actively adjust any decrease in cerebral perfusion pressure by vasodilatation and adjust for increased BP (55, 61) .

The more functional disturbances associated with T1DM induced stroke include hyperglycemia and hypertension mediated regional alterations in CBF (6, 10, 26). Systolic blood pressure is a regulator of CBF and hypertension causes marked adaptive changes in the CBF, which lead to increased brain vascular resistance and loss of the physiological mechanism of autoregulation observed in T1DM (61, 65). T1DM patients that experience hypertension also exhibit altered CBF, in particular an overall chronic reduction in CBF (68). A chronic reduction of CBF carries increased risk factors for a multitude of cerebrovascular diseases and it is clear that an absolute reduction in CBF is a high risk factor for susceptibility to stroke (26). Brennan et.al established that chronic hyperglycemia is associated with a reduction in global CBF (gCBF), the same effect observed with acute hyperglycemia in animal models (5, 6, 21). Subsequent investigations illustrated a more regional alteration in CBF and a linear relationship between the degree of hyperglycemia and the decrease in gCBF (53, 70).

Endothelial dysfunction is an injurious vascular manifestation of hyperglycemia and hypertension. The mechanism(s) by which ED arises is initiated by hyperglycemia mediated imbalance in NO bioavailability and subsequent accumulation of ROS (58). Specifically, toxic production of NO leads to an accumulation of superoxide anion ( $O_2^-$ ) and the subsequent formation of the ROS, peroxynitrite ( $ONOO^-$ ) (4, 22, 35). Peroxynitrite is a potent oxidant that initiates substrate nitration and protein nitrosylation which terminally attenuates the activity of eNOS (4). This reduced eNOS activity further renders the vascular environment vulnerable to reduced NO-dependent vascular responses and promotes key steps in vascular inflammation during stroke injury,

including increased leukocyte adhesion to endothelial cells, recruited platelet aggregation and underlying smooth muscle proliferation (58, 65).

During an ischemic event, inflammatory regulators contribute to and exacerbate stroke injury (65). At the site of focal ischemia, there is an increased aggregation of neutrophils, monocytes and transmigration of adhesion molecules, which subsequently triggers cytokine signaling and recruits pro-inflammatory cytokines to the site. Cytokine signaling recruits tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6) and iNOS to the ischemic site and elevates the inflammatory response causing exacerbated stroke injury. T1DM may exacerbate the sequelae of such ischemic events leading to greater brain edema and larger infarct volume (36, 46).

Perturbations to NO regulation are detrimental to vascular homeostasis, particularly endothelial function. It is known that physical activity enhances endothelial function through adaptive changes in NO regulation, particularly through eNOS mediated interactions (57). NO is synthesized by three NOS isoforms that include neuronal (nNOS, NOS-1), inducible NOS (iNOS, NOS-2) and endothelial NOS (e-NOS, NOS-3) (22). nNOS is expressed in specific neurons in the CNS whereas iNOS, primarily identified in macrophages, can be stimulated by cytokines in a variety of tissues, especially in inflammatory disease (22). eNOS confers vascular protection throughout the system via constitutive production of NO which then allows the vasculature to carry out physiological vasodilation, in optimal and sub-optimal stress conditions (18, 57). A baseline level of NO is necessary for optimal vascular function, however toxic levels of NO produced by iNOS, shifts the excess NO into a separate biological pathway resulting

in an accumulation of superoxide anion and the production of harmful oxidants, such as peroxynitrite (7, 57).

### **1.5 Exercise Physiology & Implications in T1DM**

Physically active organisms have a better tolerance to various stressors when compared to their sedentary counterparts, due to a progressive systemic pre-conditioning (78). Exercise is a relatively minor physiological stressor which nonetheless may initiate a stress response if the exercise intensity is sufficient (47). This may elicit protective downstream adaptations that are cell, condition and system specific (8). These adaptive responses are also specific to the exercise modality, intensity and duration (38, 48). Aerobic exercise training has been illustrated to be beneficial for reducing all-cause risk of premature death in populations highly susceptible to cardiovascular and cerebrovascular diseases, including models of aging and T1DM (2,50,24). Exercise preconditioning studies in ischemia-reperfusion models in the myocardium have illustrated an exercise-training induced ischemia tolerance, or attenuation of post-ischemic consequences (49, 72). Likewise, there has been a progressive movement in understanding the endogenous neuroprotective effect underlying brain ischemia tolerance in models of ischemic stroke, reperfusion injury and recovery (81).

Neurodegenerative therapeutics have suggested a neuroprotective role of higher intensity aerobic exercise training in potentially decreasing the risk, delaying the onset and/or alleviating symptoms exhibited in a number of neurodegenerative diseases (2, 3, 10, 50, 69). According to research conducted in which the middle cerebral artery is briefly occluded, (MCAO), the extent of cerebral consequence is shown to be directly affected by the severity of ischemic insult and by the post-ischemic response, which

includes activation of pro-inflammatory cytokines (36, 44). Kitagawa et al. established the phenomena of 'brain ischemia tolerance' wherein pre-conditioning events, such as high intensity exercise, attenuated post-stroke related injury (11, 81). The attenuated stroke injury is a result of decreased activation of pro-inflammatory cytokines (such as IL-6 and TNF $\alpha$ ) and iNOS, in conjunction with an attenuated regional and significantly lowered cerebral infarct volume (17, 41, 81). Ding et al. have demonstrated an aerobic exercise-preconditioning induced reduction in cerebral infarct volume and suggest this reduction observed can be attributed to vastly improved post-stroke recovery mechanisms (13, 17).

Focal ischemia is initiated in the larger vessels however the mechanisms of stroke induction and exercise-mediated neuroprotection remains unclear and have directed current stroke research towards the core of microcirculation and vascular pathobiology. Studies with MCAO induced stroke and observations of post-stroke reperfusion hinted at the integrity of microcirculation as a determinant of the severity of consequences during ischemic reperfusion and recovery (2, 17). Therefore, a further understanding of changes in the vasculature would provide closer perspective on the potential mechanisms underlying stroke injury and the effect of exercise training.

Various cerebrovascular diseases are characterized by marked changes in CBF, in particular, a reduction of CBF (39, 61, 68). During exercise, the cardiovascular output is actively modifying systemic vascular blood flow to working regions, however global CBF (gCBF) is known to remain constant despite regional changes in CBF (39). Studies assessing middle cerebral flow velocity (MCAv) using pulsed doppler ultrasound, in an exercise training model, have illustrated a 10-25% increase in MCAv following exercise training in aged adults (53). It is important to note that aging itself is characterized by



chronic reductions in CBF. Consequently this response could be differently affected, in which case exercise could bring CBF back to baseline conditions observed prior to the onset of aging (55, 65). Though MCAv is elevated post-training, gCBF remained constant and unaffected by exercise in those particular investigations. Ainslie et al. demonstrated that there was a change in gCBF when adjusted for varying exercise intensities (55). With moderate intensity training, which equates to 60%  $\text{VO}_{2\text{max}}$ , there was marked elevation in gCBF (53, 55). In contrast, with higher intensity exercise where  $\text{VO}_{2\text{max}} > 60\%$ , there is a significant decline in gCBF to baseline levels and during that window of heavy metabolic demand, it is postulated that transient hyperventilation mechanisms take over for short-term metabolic demand (39, 55). The brain strives to maintain its autoregulation, in which case high intensity aerobic exercise generally cannot be maintained for extended durations. In some models of exercise training and induced stroke, exercise-trained animals exhibit higher regional CBF during post-stroke reperfusion and thus attenuated injury despite non-significant gCBF increases during exercise training. Perhaps this indicates that there may be various physiological adaptations of exercise training that contribute to the observed post-stroke protection (43). The exact mechanisms as to how CBF is governed and adapts with exercise training remains unclear.

## **1.6 Biological Basis of Heat Shock Proteins**

The exact underlying mechanism(s) as to how exercise imposes ischemia tolerance remains unclear, however research has recently been directed to the role of stress response adaptations as a possible factor in this regard. Heat shock proteins (HSP) are 'evolutionarily conserved' stress proteins that function as molecular chaperones and

exhibit a cytoprotectant role during physiological insults, including diseases that incite cellular stress. HSPs have also been widely studied for their anti-inflammatory role in protection against cytotoxicity. HSPs are classified by their molecular mass, ranging from the smaller HSP families such as Hsp27 to the larger HSPs that include Hsp70, Hsp60 and Hsp90. HSPs function as constitutive or inducible stress proteins that have physiological importance that are condition and tissue specific (56, 76). The constitutive HSPs are generally thought to engage in housekeeping roles while the inducible HSPs are up regulated by a stress stimulus for directed cellular protection (56).

Under physiological conditions Hsps are primarily located in the cytoplasm and bound to heat shock factors (HSFs), of which there are three variant transcription factors in mammals (54). When sufficient physiological stress leads to some protein denaturation or damage, dissociation of HSPs from HSF occurs with subsequent nuclear localization and stress protein induction occurs (54).

### **1.6.1 Molecular Basis of HSPs**

Within the chaperones, the 70-kilodalton (kDa) family of HSPs, Hsp70, has been most characterized in exercise health and disease models (54, 76). The Hsp70 family of proteins encompasses the constitutively expressed protein, Hsc70 (Hsp73), and its more widely studied inducible counterpart, Hsp70 also known as Hsp70i and Hsp72 (54). Researchers have long established the ubiquitous role of Hsp70 in the maintenance of cellular homeostasis. Under normal physiological resting conditions, Hsp70 exists in low quantities in most tissues, unlike the constitutive counterpart, Hsc70 (79). The physiological benefits of Hsp70 overexpression have been established in a variety of

systemic models. Consequently, heat shock pre-conditioning induced elevations in myocardial Hsp70 expression have been observed in conjunction with decreased ventricular infarct damage post-ischemia (40, 49). Further, Hayes et.al noted an increased Hsp70 mRNA content was directly correlated with reduced area of cerebral infarct following MCAO induced ischemic stroke (12).

The constitutively present 90-kDA heat shock protein, Hsp90, accounts for one of the most abundant cytosolic proteins (63, 64). Hsp90 functions as an interactive housekeeping protein that ensures the maintenance of protein-folding and aggregation and acts as a chaperone to many client proteins (59). Of particular interest, Hsp90 has been demonstrated to interact directly with eNOS and thus mediate the intricate balance between NOS, NO and superoxides (18, 69). Hsp90 primarily behaves as a signal transduction molecule. For example, it initiates phosphorylation of eNOS in order to regulate vascular permeability and vasodilation (69).

### **1.6.2 Physiological Basis of HSPs and Exercise**

The magnitude and duration of exercise stress plays a critical role in the stress induction response such that, It is evident, the more demanding the exercise, the more robust the HSP induction (48). With aerobic training, Harris et.al demonstrated a sustained training-induced increase in Hsp70 levels in rodent striated muscle, whereas the response was diminished in other Hsps studied. Data from Milne and Noble, illustrated only high-intensity exercise training is sufficient to induce myocardial Hsp70 when compared to lower intensity training(49). Physical activity induction of Hsp70 has not been as actively investigated in the brain as systemic tissue, however Campisi et.al demonstrated habitual exercise stress does induce a HSR and subsequent Hsp70 response

in the various regions of the brain including hypothalamus, hippocampus and prefrontal cortex alongside their respective adjacent areas (8). They postulated that the Hsp70 response could potentially be neuroprotective given observations from training studies in skeletal muscle and myocardium (8)

Hsp90 is not as extensively characterized as Hsp70 in exercise training models. Atalay et.al has observed no change in Hsp90 expression in vastus lateralis and red gastrocnemius following an 8-week aerobic exercise training intervention, which can be attributed to the specific type of muscle investigated (25). Other studies, did note an increase in Hsp90 expression in the soleus muscles and vascular increase in eNOS expression (25, 66). These observations are attributed to exercise mediated increases in NO to allow for increased flow to working muscles, resulting in increased shear stress on vascular wall (24). Hence, exercise induced changes in Hsp90 may be more associated with the vasculature than the skeletal muscle fibers. No knowledge exists in the cerebrovascular Hsp90 and eNOS expression following exercise training, however it can then be assumed that Hsp90 and eNOS would respond differently in cerebrovasculature when compared to skeletal muscle, in order to strictly regulate steady cerebral flow when total cardiac output rises.

### **1.6.3 Physiological Basis of HSPs and Disease**

A myriad of biological mechanisms have been under close scrutiny in an effort to better understand the exercise mediated ischemic tolerance in stroke recovery (16). The interaction between chaperone proteins HSPs and cellular stress response have been fundamental constituents in understanding the pre-conditioning mediated cerebral tolerance exhibited in stroke models.

Hsp70 over-expression produces notable physiological benefits in many tissues. More recently, it includes potential anti-inflammatory actions regulated by Hsp70. Wieten et. al demonstrated Hsp70 up regulation with concurrent inhibition of inflammatory cytokines that include TNF $\alpha$  and IL-6 (79, 82). Recent studies by Weiss et.al have established that direct interaction between Hsp70 and an inhibitor of nuclear factor kappa-B kinase subunit alpha (IKK- $\alpha$ ), interrupts the activation of a master pro-inflammatory transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) (43, 82). Co-immunoprecipitation studies by Zheng et.al have illustrated an Hsp70 mediated inhibition of NF- $\kappa$ B and associated NF- $\kappa$ B regulatory genes that specifically include pro-inflammatory expression of iNOS (82). .

The stress response has a differential impact on constitutive proteins when compared to their inducible counterparts. Variability in Hsp90 expression has been implicated in regulation of oxidative stress in pathological conditions such as hypoxia and preeclampsia (57, 63). In T1DM animal models, the progressive hyperglycemic state appears to elicit a dissociation in the Hsp90 interaction with eNOS which has been attributed to an increased interaction between Hsp-90-IKK $\beta$  and deactivation of eNOS (63). In effect, increased Hsp90-IKK interactions allow for subsequent activation of NF $\kappa$ B and the ensuing inflammatory and cytokine response while simultaneously blunting NO signaling (51).

## **1.7 Nitric Oxide Synthase and HSPs**

### **1.7.1 iNOS and Hsp70**

Type one diabetes is a major risk factor for vascular dysfunction whereby altered NO regulation and elevated oxidative stress leads to vascular inflammation and ischemic

stroke. iNOS is a pro-inflammatory protein, that upon induction serves as a primary inflammatory agent, eliciting the synthesis of toxic levels of NO (35). Immune reactions are mediated in part through activation of NF- $\kappa$ B through physiological stress (including ischemia and oxidative stress), leading to an up regulation of downstream pro-inflammatory genes including iNOS (44). (Fig.1) Under normal physiological conditions, there exists low to no CNS expression of iNOS however during inflammatory events, iNOS is induced by cytokines that include TNF $\alpha$ , Interferon gamma (IFN $\gamma$ ) and Interleukin-1 beta (IL-1 $\beta$ ) (30, 35). This immunological response is observed and exacerbated in T1DM brain, during ischemic stroke (43). Parmentier et.al demonstrated the impact of exogenous administration of iNOS blockers on the reduction of post-ischemia cerebral infarct volume (44). Ross et.al illustrated a considerable reduction in infarct volumes in an iNOS knockout mouse model compared to wild-type animals, when subjected to a bout of cerebral ischemia (62).

Hsp70 is involved in adaptive and innate immune regulation which varies according to the location of its induction and expression (82). Intracellular overexpression of Hsp70 in astrocytes has been noted to reduce inflammatory induction of toxic levels of NO by iNOS (79). As mentioned, physiological levels of NO derived from eNOS governs normal vasodilation in the system, however toxic levels of NO oftentimes 10 times as much as eNOS produced by iNOS, results in peroxynitrite formation and free radicals causing heightened oxidative stress. Hsp70 overexpression directs an inhibition of inflammatory responses through deactivation of NF- $\kappa$ B and the downstream NF- $\kappa$ B dependent pro-inflammatory component, iNOS (79, 80, 82). In effect, Hsp70 elicits protection through inhibition of pro-inflammatory proteins that

would otherwise compromise brain health in various conditions of inflammation. Induced or transgenic overexpression of Hsp70 correlate to a significant reduction in brain infarct volume following ischemic insult (41). The exact mechanism of this antagonistic interaction, where an overexpression of Hsp70 is noted with a concurrent down regulation of iNOS, is not entirely clear. Yenari et.al, implemented a transient MCAO stroke model in transgenic mice exhibiting Hsp70 overexpression, through which the preliminary data illustrated increased Hsp70 association with NF- $\kappa$ B and I $\kappa$ B during reperfusion and recovery (79, 80). This increased association then prevents phosphorylation of I $\kappa$ B by IKK and consequently establishes the inhibition of NF- $\kappa$ B (51, 82). In doing so, Hsp70 overexpression illustrated to regulate the inhibition of NF- $\kappa$ B and subsequent downstream inflammatory components. Data from Yenari et.al allude to an Hsp70 mediated anti-inflammatory regulation and an inhibition of iNOS, which would warrant further characterization of Hsp70 and iNOS in disease conditions that are threatened by inflammatory consequences (80, 82).

### **1.7.2 eNOS and Hsp90**

From its discovery, Hsp90 has been established as a regulator of varying signal transduction pathways that include interactions with over 200 client proteins and 50 co-chaperones, making it difficult to characterize its primary role (59). Investigations evaluating more specific Hsp90 interaction with cellular constituents provide a better understanding of the role of Hsp90 in health and disease. In the latter 1990s, Sessa et.al introduced the biological association between Hsp90 and eNOS, whereby his team of researchers demonstrated activation of eNOS upon binding with Hsp90 (63). In doing so, Hsp90 interacts with eNOS and upon interaction Hsp90 is further recruited to the

eNOS complex by surrounding molecular agonists (69). Vascular regulation relies on eNOS, which governs blood flow, blood pressure and angiogenesis through physiological NO production (63, 64). Exercise training results in elevated systemic vascular eNOS expression, localized to endothelium and VSMCs, in conjunction with increases in concentration of NO (67). This is postulated to be due to exercise-induced shifts in laminar flow and shear stress on the vasculature, in order to ensure adequate perfusion to the working system. From a T1DM perspective, Mohan et.al illustrated an IKK $\beta$  interaction with Hsp90 and this interaction is augmented by hyperglycemia in cultured vascular endothelial cells, in doing so this diminishes Hsp90 interactions with eNOS (51). This diminished interaction is one possible mechanism that illustrates how severe hyperglycemia may attenuate eNOS activation and expression, observed in the progression of T1DM phenotype. Fig. 1.

### **1.8 The T1DM Insulin HSP Connection**

Individuals with T1DM undergo exogenous insulin therapy, which incorporate subcutaneous planted insulin pumps or repeated injections to maintain physiological blood glucose concentrations (3). In the brain, insulin levels are governed by the endothelial transport across the blood-brain barrier into the cerebrospinal fluid and interestingly, the cerebral levels are noted to be independent of plasma insulin, however systemic changes in circulating insulin still govern cerebral functions (3). Acute studies noted a short-term insulin withdrawal lead to deleterious neurochemical changes in brain and subsequent insulin replacement lead to complete or partial restoration of cerebral function (6). Notably, T1DM is a condition that is initiated through an autoimmune targeted elimination of pancreatic beta cells and or circulating insulin (3). Investigations



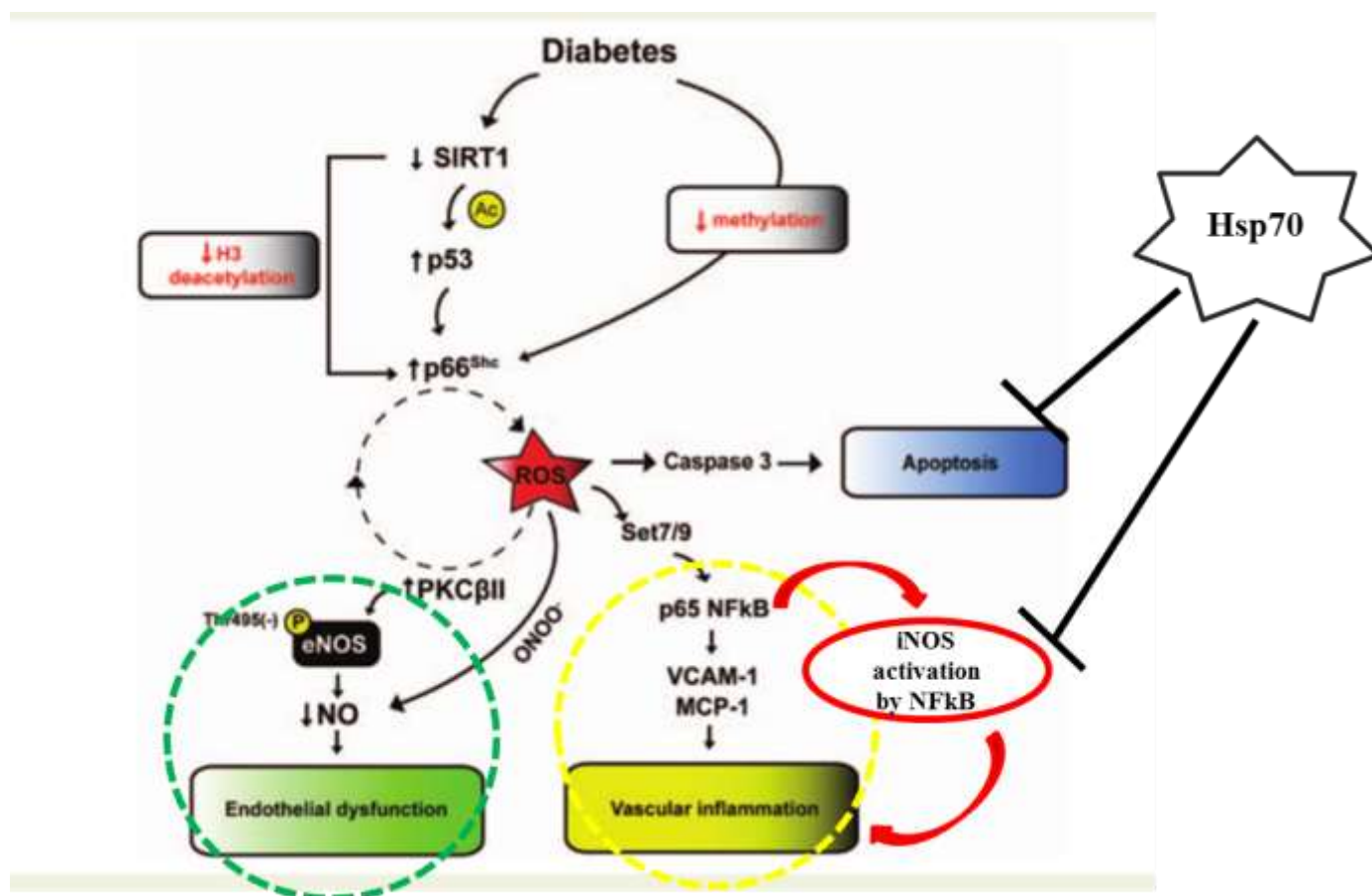
using bacterial Hsp70 orthologs hint at the depletion of Hsp70 as possible mechanism to the observed increase in epitope recognition by T cells and subsequent acceleration in the immune-mediated progression of T1DM (73). With studies investigating diabetic peripheral neuropathy (DPN) it is then postulated co-administration of other diabetic treatment including heat shock inducers maybe perhaps improve or attenuate the progression of DPN (73). Studies by Li et al have illustrated an insulin mediated induction of low-level vascular Hsp70 expression alluding to a possible shared signaling pathway between Hsp70 and insulin (40).

## **1.9 Summary**

T1DM is a risk factor for stroke, of which ischemic stroke accounts for 80-85% of stroke incidence. Hyperglycemia mediated metabolic shifts such as an increase in oxidative stress in the microcirculation, have been implicated as a mechanism by which diabetes contributes to a significant increase in the risk for vascular dysfunction in the brain. Epidemiological studies with exercise pre-conditioning in humans, have illustrated increased brain ischemia tolerance correlated to a decrease in post-stroke injury and/or the potential to decrease the prevalence of ischemic stroke. It has been illustrated that exercise preconditioning stress confers neuroprotection from ischemic stress in conjunction with an increased tissue HSP induction. Of particular interest, Hsp70 overexpression has been attributed to the neuroprotection in conditions of both focal and global ischemia. This overexpression of Hsp70 has been attributed to increased brain ischemia tolerance and has also been linked to anti-inflammatory role, specifically an Hsp70 mediated inhibition of NF- $\kappa$ B and iNOS. In models of health and disease, the function of housekeeping regulation is oftentimes overlooked due to their highly

regulated constitutive nature. However it is important to elucidate the interactions of housekeeping proteins with secondary components. Hsp90 interactions with eNOS highlight the potential importance of protein-interactions in disease, explicitly the progression of diabetes induced vascular dysfunction. Diabetes induced endothelial dysfunction has been linked a decreased expression of eNOS and exercise training has differentially affected this response in systemic tissues. Patients with T1DM, despite exogenous insulin therapy, still experience progressive microcirculatory insults from metabolic glycemic shifts. Pharmacological HSR inducers have recently become a potential therapeutic thought however very little is still known of the cerebral heat shock response and the characterization of diabetic stress in cerebrovasculature. Exercise training remains a viable potential therapeutic strategy for diabetes induced cerebrovascular complications. Thus the objective of this study is to primarily evaluate the effect of 10-weeks of high intensity aerobic training alone and in controlled to slight hyperglycemic T1DM condition, on HSP and NOS expression in large and small cerebral vessels. The co-localization of Hsp70, Hsp90, iNOS and eNOS proteins with vascular smooth muscle cells, will in addition help elucidate morphological characterization of the proteins of interest in cerebral vessels. It is hypothesized that a 10-week high intensity training protocol will be associated with a long-term robust increase in Hsp70 expression and T1DM could potentially blunt this training response observed in the trained only group. Secondly, it is hypothesized that following diabetes induction with no exercise training intervention, there will be an increase in iNOS expression in the sedentary T1DM group. Lastly, it is hypothesized that exercise training will be associated with a

relative increase in Hsp90 and eNOS expression, when compared to the non-trained groups.



**Fig.1.1 Nitric Oxide Regulation in Diabetes**

Green dashed encapsulation highlights eNOS bioactivity on endothelial dysfunction. Yellow dashed encapsulation highlights NF-κB bioactivity with the downstream regulation of iNOS, and how it all collectively contributes to the development of vascular inflammation. Visual representation of the anti-inflammatory contributions of Hsp70 overexpression, through inhibition of iNOS.

Figure adapted from Paneni et.al.2013(58)

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## CHAPTER 2

### 2.1 Introduction

Strokes are the second leading cause of death worldwide and ischemic stroke accounts for over 80% of the incidents (9). Epidemiological studies confirm a high risk for ischemic stroke in patients with Type 1 Diabetes Mellitus (T1DM) (11, 31). T1DM is an autoimmune disorder caused by the immune mediated destruction of pancreatic  $\beta$  cells, resulting in an absolute decline in the production of insulin and in increased level of circulating glucose (36). Hyperglycemia is a major atherogenic factor underlying the macro- and microvascular complications of Type 1 diabetes that evolve to become primary contributors to injurious cardiovascular events, stroke, or peripheral vascular disease (28, 30). The majority of ischemic stroke incidents are a terminal causality of endothelial dysfunction and accelerated atherosclerosis (17, 42).

Aerobic exercise is a physiological stressor, established as a preventative tool in reducing risk factors for cardiovascular and cerebrovascular complications (7, 36). Aerobic exercise training has been attributed to the neuroprotective phenomena of 'brain ischemia tolerance' (38). It is suggested that pre-conditioning exercise stress builds an adaptive tolerance in the brain by strengthening the recovery response post-ischemic event and in effect attenuating the degree of cerebral damage observed (2, 38).

Exercise functions as a physiological stimulus which elicits a systemic stress response that includes the heat shock response (HSR). This response has a downstream impact on a hierarchy of molecular regulators, such as heat shock proteins (HSPs) (12, 29). HSPs are a family of chaperone proteins that are constitutively present and stress inducible and which function as tissue-specific cytoprotectants against various

pathological conditions. The inducible 70kDA HSP (Hsp70), is a stress induced molecular chaperone that offers cardioprotection and is up-regulated with exercise (22, 26, 27). Recently, these protective Hsp70 proteins have been found to increase in skeletal muscle vasculature following exercise where they likely reduce oxidative stress and protect endothelial integrity and vascular signaling (6, 26). In the brain, increased Hsp70 tissue mRNA correlates to a reduced area of brain death, observed in a middle cerebral artery occlusion (MCAO) stroke model (14, 20). Studies in T1DM models, hint at HSP associated neuroprotection against severe ischemic strokes whereby overexpression of Hsp70 has been noted to curb the severity of focal and global cerebral ischemia via attenuated post-stroke infarct volume (37, 39). In an experimental T1DM model, Lappalainen et.al recently illustrated a blunted Hsp70 response in whole brain tissue lysates following chronic exercise training in diabetic trained when compared to non-diabetic trained (20). The distribution and up-regulation of Hsp70 in cerebral vasculature is less clear, particularly following training and in chronic disease conditions such as diabetes.

Hsp70 functions as part of a multi-chaperone complex with co-chaperones, such as the 90kDA HSP(Hsp90). Hsp90 is a constitutively present cytosolic protein that promotes the vasodilation of blood vessels via interactions contributing to the phosphorylation of endothelial nitric oxide synthase (eNOS), which produces nitric oxide (NO) to increase vascular permeability and promote vasodilation (13, 33). In a resting state, Hsp90 is associated with eNOS. Upon stimulus of the cells from varying stressors such as hormones, shear stress or statins, the association between the two proteins increases, resulting in physiological NO production (13, 23). One of the hallmarks of

Type I diabetes is reduced vascular function (5). However, it is unknown whether changes in Hsp90 in the cerebral vasculature are negatively affected by diabetes. The influence of exercise training on this protein in cerebral vasculature has not been examined either (25). Previous studies, with chronic and acute aerobic training, noted an increase in Hsp90 expression in the soleus muscles and vascular increases in eNOS expression (25, 66). These observations suggest exercise mediated increases in NO to allow for increased flow to working tissues (13, 21). Given the differing blood flow changes that occur with exercise in the vasculature of skeletal muscle versus the brain, the response of Hsp90 and eNOS to exercise training in the latter is unclear.

Endothelial dysfunction (ED) exhibited in T1DM has been linked with an imbalance in NO content and concurrent shifts in nitric oxide synthase (NOS) regulation (15, 16). The NO balance is critical for cellular function and is maintained by physiological production of NO by eNOS (10, 32). The onset of NO imbalance can be attributed to toxic over-production of NO, by inducible NOS (iNOS) in which case iNOS produces 10 times as much NO as eNOS derived NO. This overproduction stimulates inflammatory cascades and shunts the excessive NO towards the formation of superoxide anion ( $O_2^-$ ) and the accumulation of the reactive oxygen species (ROS) and peroxynitrite ( $ONOO^-$ ). Peroxynitrite is a potent oxidant which initiates substrate nitration and protein nitrosylation and terminally attenuates the activity of eNOS (4, 32). In T1DM there is a decreased bioactivity of eNOS and in particular cases, that response is coupled with an increase in iNOS bioactivity (1, 32). Mohan et.al demonstrated that Hsp90 interacts with an inhibitor of NF- $\kappa$ B, I $\kappa$ B kinase  $\beta$  (IKK $\beta$ ) and that this interaction, which is augmented by hyperglycemia in cultured vascular endothelial cells, diminishes Hsp90

interactions with eNOS (31). This diminished interaction between eNOS and Hsp90 is one possible mechanism that explains the decreased eNOS bioactivity in T1DM. This reduction may render the vascular environment vulnerable to reduced NO-dependent vascular responses and promotes vascular inflammation during stroke injury(34).

Potential mechanisms underlying improved post-stroke recovery have been attributed to exercise preconditioning mediated inhibition of pro-inflammatory factors including tumor necrosis factor alpha (TNF $\alpha$ ), nuclear factor  $\kappa$  B (NF- $\kappa$ B) and iNOS(8). The overexpression of Hsp70 has been attributed an anti-inflammatory role, specifically an Hsp70 mediated inhibition of NF- $\kappa$ B and its downstream factor, iNOS(1, 18, 40).

The purpose of this study is to evaluate the effect of 10-weeks of high intensity aerobic training alone and in a controlled slight hyperglycemic T1DM condition, on HSP and NOS expression in large and small cerebral vessels. The method evaluating the hypothesis requires independent localization of Hsp70, Hsp90, iNOS and eNOS proteins; thus the study will elucidate morphological localization of the proteins of interest in cerebral vessels. It is hypothesized that a 10-week high intensity training protocol will be associated with a significant increase in Hsp70 expression and T1DM could potentially blunt this training response observed in the trained only group. Secondly, it is hypothesized that, following diabetes, there will be an increase in iNOS expression in the sedentary T1DM group. Lastly, it is hypothesized that exercise training will be associated with a relative increase in Hsp90 and eNOS expression, when compared to the non-trained groups.

The findings of this study has the potential to direct further attention to the exercise induced vascular protection that can be translated into interventions in chronic diseases that include components of cerebral vascular pathology, such as T1DM.

## **2.2 Methods**

### *Ethics approval*

This study was approved by the Research Ethics Board of the University of Western Ontario, which is in accordance with the guidelines of the Canadian Council on Animal Care (Appendix B.1).

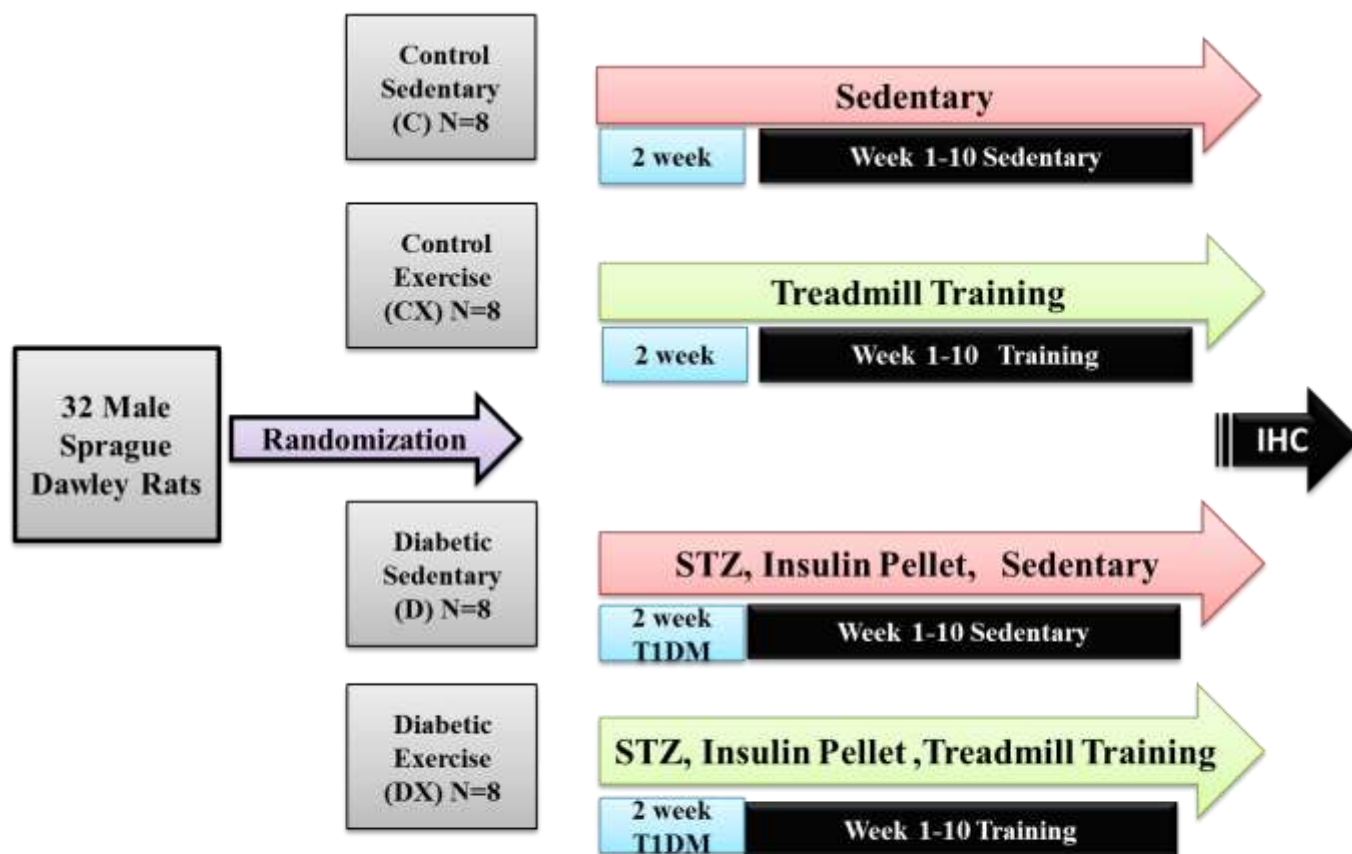
### *Animals*

Sixty-four, 8-week old, male Sprague-Dawley rats were obtained from Charles River Laboratories (St. Constant, QC, Canada). The animals were housed as cage pairs, sustained on a 12-hour dark/light cycle at a constant temperature ( $20 \pm 1^{\circ}\text{C}$ ) and relative humidity (50%) throughout the entirety of the experiment. All of the animals were allowed access to standard rat chow and water *ad libitum*.

### *Experimental Groups*

The rats were randomly divided into one of four treatment groups of eight animals each. The resulting groups are the following: control sedentary (C; n=8), control exercised (CX; n=8), diabetic sedentary (D; n=8) and diabetic exercised (DX; n=8). (Figure 2.1)





**Fig 2.1 Experimental Study Design**

Experimental setup illustrates the relative timeline, individual to each animal group understudy.

### *Experimental Procedures*

#### *Diabetes Induction and Insulin Implantation*

To enable a more translational approach to studying T1DM, a recently established model of controlled slight hyperglycemia with insulin was employed for this study (25). This particular methodology incorporated multiple low-dose streptozotocin (STZ) treatment for the immune induction of diabetes (35) with a clamping of blood glucose levels to between 9 and 15mmol/L blood glucose (Appendix A1, A2). The multiple low-dose STZ induction of diabetes consisted of 5 consecutive days of intraperitoneal

injections of STZ (20mg/kg), which was dissolved in a cocktail of citrate buffer (0.1M, pH 4.5). The insulin therapy was provided by subcutaneous implantation of exogenous insulin pellets (1 pellet; 2U insulin/day; Linplant, Linshin Canada, Inc., Toronto, Ontario, Canada) (Appendix A2).

The disease state was confirmed with two measures of non-fasted blood glucose (BG)  $\geq 18$ mmol/L, following the 5-day induction of STZ. Subsequent injections were conducted until the diabetic state was confirmed.

### *Insulin Dosage*

The state of slight hyperglycemia (non-fasting BG 9-15mM range) in D and DX animals was maintained throughout the study with insulin pellet adjustments. (Appendix A2).

Following the completion of study, the dosage of insulin was measured by multiplying the total number of pellets implanted by the units of insulin secreted per pellet. The resulting measure was then individually divided by the animal's body weight to obtain the relative circulating insulin dosage.

### *Aerobic Exercise Training Protocol*

Exercise training began 2 weeks after induction of diabetes in the diabetic animals and at an age-matched time point for their sedentary controls. Rats were familiarized on a motor-driven treadmill, the week prior to the onset of their 10-week training protocol. The familiarization protocol consisted of 15 m/min for 5 minutes, 21 m/min for 5 minutes and 27 m/min for 5 minutes. Puffs of air and tactile stimulation were used to encourage the animals to run. The 10-week training regimen incorporated 60 minutes of running, 5 days a week and at exercise speeds of 27 meters per minute at a 6° incline. The intensity

of the aerobic exercise, 27m/min was pre-determined to represent higher intensity aerobic at approximately 70%-75%  $\text{VO}_{2\text{max}}$  (35).

### *Experimental Measures*

#### *Body Weights, Blood Glucose, Blood Pressures*

Body weights (BW) and blood glucose concentrations (BG) were measured at week 1, week 6 and week 10 of the training period. Blood (approximately 50 ul drop) was collected from the saphenous vein and blood glucose concentration assessed via the Freestyle Lite Blood Glucose Monitoring System (Abbott Diabetes Care Inc., Mississauga, Ontario, Canada).

#### *Exercise Temperatures*

For the two groups of animals that underwent exercise-training, CX and DX, pre- and post-exercise rectal temperatures were taken at mid-point (Week 6) of training and at the endpoint (Week 10). Additionally, endpoint rectal temperatures were collected from sedentary groups, C and D.

#### *Tissue Collection*

At the end of the experimental period animals were anesthetized with urethane  $\alpha$ -chloralose and euthanized by exsanguination. Exercise-trained animals were anesthetized 72 hours following their last exercise session in order to avoid any acute exercise changes in tissue morphology. Whole brains were extracted from the cranial cavity, placed in 4% paraformaldehyde (PFA) (Fischer Scientific, Corp.), which was renewed daily for 3 days and then placed in 70% ethanol for temporary storage.

### *Tissue Processing*

Prior to fluorescence immunohistochemistry, whole fixed brains were split transversely and embedded in paraffin. The paraffin blocks were transversely cut in serial 10µm thick sections using a microtome (Microm HM33E, Leica). (Appendix A3) Sections were mounted on frosted, poly-L-Lysine-coated, positively charged microscope slides (VWR® Superfrost® Plus Micro Slide 48311-703, 25x75mm) and stored at room temperature for Hematoxylin & Eosin (H & E) and fluorescence immunohistochemistry. Each slide held two serial sections of the tissue in order to have negative controls.

### *Histochemical Stains*

The vascular targets in this study included superior cerebellar vessels adjacent to the entorhinal cortex. In order to observe and confirm histological structures of interest, sections were deparaffinised (Leica autostainer XL) and stained with a hematoxylin (H&E) protocol using a Leica autostainer XL (Leica ST5010). (Appendix A4)

In order to observe vessels versus adjacent nerve matter, sections were deparaffinised and stained with Luxol fast blue overnight at 58°C, dehydrated in 95% and 100% clear alcohol, followed by two changes in xylene and cover slipped. (Appendix A4)

### *Fluorescence Immunohistochemistry (IHC)*

As per Maddahi et.al (24) sections were further analyzed using a 2-day, immunohistochemical protocol.

Day 1: Sections were deparaffinised on the autostainer and antigen retrieved for 2 hours (Pick Cell 2100 with sodium citrate buffer [10mM sodium citrate, 0.05% tween-20, pH 6.0]), following which the slides were transferred to glass coplin jars filled with

phosphate buffered saline (PBS, 1M). Slides were then dried with Kimwipe and individual tissue sections on the slide were circled with a grease pen (Calbiochem ImmunoPen™, EMD Biosciences) and placed in humidity chambers. Slides were blocked at room temperature with 10% goat serum in PBS for 1 hour. In the meantime, primary antibodies were thawed at room temperature and assembled as cocktail dilutions in 1% Tween-PBS (TPBS) goat serum, which allowed for co-localization of VSMC with proteins of interest, Hsp70, Hsp90, iNOS and eNOS. The primary antibodies were combined as follows:

Concentrations and combinations are detailed in Table 2.1:

Table 2.1: Antibody Dilutions

1° Antibody Cocktails	Antibody Identifier	Antibody Concentration
<b><i>Hsp70 + Smooth Actin</i></b>	Hsp70 (SPA-812)	1:100
	Smooth $\alpha$ actin (AB18147)	1:400
<b><i>Hsp90 + Smooth Actin</i></b>	Hsp90 (AB13485)	1:400
	Smooth $\alpha$ actin (AB18147)	1:400
<b><i>iNOS + Smooth Actin</i></b>	iNOS (AB 15323)	1:100
	Smooth $\alpha$ actin (AB18147)	1:400
<b><i>eNOS + Smooth Actin</i></b>	eNOS 1:100 (BD610299)	1:100
	Smooth $\alpha$ actin (AB18147)	1:400

Following blocking, slides were washed in PBS for three cycles of 5 minute increments and dried with Kimwipes. One out of two possible tissue sections on every slide were incubated with primary antibodies overnight at 4°celsius. The remaining section was

incubated with 1% TPBS goat serum in the absence of primary antibodies in order to function as a negative control to test for non-specific staining.

Day 2: The slides were washed in PBS for three cycles of 5 minute increments and dried with Kimwipes, following which all sections were incubated with a cocktail of secondary antibodies for 1 hour at room temperature.

Secondary Antibody Cocktail: Anti-mouse (Alexa Fluor®488 Goat Anti-Mouse IgG, Invitrogen, 1:400) and anti-rabbit secondary (Alexa Fluor®594 Goat Anti-Rabbit IgG, Invitrogen, 1:400) antibodies in 1% goat serum in T-PBS.

Following secondary incubation slides were washed in PBS for three cycles of 5 minute increments and dried with Kimwipes. For the purpose of fluorescent imaging, slides were mounted with a fluorescent mounting medium (Prolong® Gold Antifade Reagent containing DAPI, Invitrogen) containing 1% 4', 6-diamidino-2-phenylindole (DAPI), a fluorescent nuclear stain and then covered with glass cover slips (VWR® SuperSlips™ Micro Cover Glasses, Rectangular, No. 1, 24 X 55mm, CA48382-128). In order to maintain the fluorescent signal integrity and minimize photo bleaching, the slides were stored in a slide folder at 4°C until imaging. (Appendix A6)

#### *Fluorescent Microscopy and Protein Abundance Acquisition*

The fluorescent bright field microscopy was carried out on a Zeiss Axiovert S100 microscope and images were captured at 16X and 40X magnification, using Northern Eclipse 6.0 (Empix Imaging Inc) software. The protein content/abundance signal for Hsp70, Hsp90, iNOS and eNOS were extracted using Image J (NIH). Specifically, protein content was determined by outlining the wall of the blood vessel of interest and calculating the integrated pixel density (signal) (Appendix A7) and the same technique

was used to determine the integrated pixel density for the negative control of the same blood vessel. To obtain the relative protein density, the pixel density of the negative control was subtracted from the pixel density of the experimental sample. The difference in pixel density provided values that were used to indicate fluorescence signal intensity and thus expression of protein. For the collection of data for both small and large vessels, this procedure was repeated for four blood vessels from the same animal and averaged to give a single value for the particular animal.

#### *Smooth Muscle Area Calculations*

For comparative analysis of vessel size and protein content, smooth muscle area (SMA) was calculated for each vessel for which the protein content was calculated. These SMA calculations allowed for protein content adjusted to the area. For SMA calculations, two full length perpendicular diameters from the outer perimeter of each vessel were averaged to obtain a diameter for the outer circle. Likewise, two perpendicular diameters from the inner 'endothelial' perimeter of each vessel were averaged to obtain a diameter for the inner circle. The area of the smaller circle area was then subtracted from that of the larger circle in order to obtain the SMA for that specific vessel. (Appendix A8)

#### *Data Analysis*

For all statistical findings, the reports were performed using SigmaPlot 11 (Systat Software Inc.) statistical software. Body weight, blood glucose concentrations, arterial blood pressures and insulin dosage values were compared across all groups using a two-way repeated measures analysis of variance (ANOVA). Rectal temperatures across the exercise-trained groups were compared using a two-way ANOVA. Where necessary,

comparative pairwise analysis was performed using either Tukey's or Dunn's post-hoc test with significance set at  $p < 0.05$ . Protein signal content data (expression) from fluorescent IHC, unadjusted or adjusted for SMA, were compared using a three-way ANOVA across all four groups. Where necessary, comparative pairwise analysis was performed using Fischer's post-hoc test with significance set at  $p < 0.05$ . A three-way ANOVA was performed to evaluate the protein content with respect to potential interactions between disease, exercise training and vessel size.

## 2.3 Results

### *Baseline Conditions*

**Body Weights:** Over the course of the study, all animals across the groups gained a significant amount of weight ( $p < 0.05$ ). Compared to diabetic sedentary (D) and diabetic exercised groups (DX), the control sedentary (C) and control exercised groups (CX) had significantly greater body weights by the end of week 10 ( $p < 0.05$ ). There was no significant effect of exercise on body weight. (Table 2.2)

**Blood Glucose:** Weekly blood glucose concentrations for diabetic groups, D and DX post-diabetes induction, were significantly higher when compared to both control groups, C and CX ( $p < 0.05$ ). There was no significant effect of exercise on blood glucose concentrations. (Table 2.2)

**Insulin Dosage:** Total insulin dosage comparison within diabetic sedentary and diabetic trained groups, D and DX, suggests that there was pattern of decreased exogenous insulin dosage in DX however, the difference was not significant ( $p = 0.061$ ).



Exercise temperatures: In the control exercised group (CX), post-exercise temperatures were significantly elevated when compared to pre-exercise temperatures, in both week 6 and week 10 measures ( $p < 0.001$ ). (Table 2.3)

*Effect of Diabetes and Training on Protein Signal Content & Localization*

Visualization of large cerebral vessels and protein signal density in the cerebral cortex is demonstrated across Control (C), Control Exercise (CX), Diabetic Sedentary (D) and Diabetic Exercised (DX) groups (Plates 1,3,5,7). In addition, the double immunofluorescence was conducted in order to achieve co-localization of protein to VSMCs across all groups. (Plate 2,4,6,8)

Hsp70: Overlay of protein to VSMCs illustrates Hsp70 signal density is primarily localized to the SMA. (Plate 1,2) There is a main effect of training on Hsp70 expression ( $p < 0.05$ ). The signal content was greater in larger vessels when compared to small vessels. (Fig.2.2) When adjusted for SMA, there is still a main effect of training on Hsp70 expression ( $p < 0.001$ ). (Fig.2.3)

Hsp90: Overlay of protein to VSMCs illustrates Hsp90 signal density is primarily localized to the SMA. (Plate 3,4) There is no main effect of exercise in the Hsp90 expression ( $p > 0.05$ ). The signal content was greater in larger vessels when compared to small vessels. (Fig. 2.4) There is a main effect of vessel size ( $p < 0.05$ ). When adjusted for SMA, there is still no main effect of training on Hsp90 expression ( $p > 0.05$ ). (Fig. 2.5)

iNOS: Overlay of protein to VSMCs illustrates iNOS signal density is primarily localized to the SMA. (Plate 5, 6) There was a main effect of disease and training on iNOS expression ( $p < 0.05$ ). The signal content was greater in larger vessels when

compared to small vessels (Fig2.6). When adjusted for SMA, there is still a main effect training and disease on iNOS expression ( $p<0.001$ ). (Fig 2.7)

eNOS: Overlay of protein to VSMCs illustrates eNOS signal density is primarily localized to the SMA. (Plate 7,8) There is no main effect of exercise in the eNOS expression ( $p>0.05$ ). The signal content was greater in larger vessels when compared to small vessels (Fig 2.8). There is a main effect of vessel size ( $p<0.001$ ). When adjusted for SMA, there is still no main effect of training on eNOS expression ( $p>0.05$ ). (Fig. 2.9)

#### *Effect of Diabetes and Training on Smooth Muscle Area*

Because vascular dysfunction may be associated with intima hypertrophy we examined the SMA in large and small cerebral vessels across the experimental conditions. There were significant differences in SMA when comparing small versus larger cerebral vessels (Fig 2.10;  $p<0.001$ ), however there were no significant differences in SMA between groups (Fig 2.10;  $p>0.05$ ).

Table 2.2. *Baseline Body Weights, Blood glucose concentration and Insulin Dosage across control sedentary, control exercised, diabetic sedentary and diabetic exercised groups.*

Groups	Body Weights (Kg)			Blood Glucose Concentrations (mm)			Insulin Dose Dose/kg
	Week 1	Week 6	Week 10	Week 1	Week 6	Week 10	
<b>Sedentary Control (C)</b>	381±20	486±40	546±48	4.5±0.38	4.6±0.45	4.0±0.40	
<b>Control Exercised (CX)</b>	354±24	447±28	510±36	4.26±0.27	4.0±0.45	3.9±0.34	
<b>Sedentary Diabetic (D)</b>	302±21*	422±25*	455±33*	10.8±3.4†	15.5±2.4 †	16.8±2.6 †	5.56±1.5
<b>Diabetic Exercised (DX)</b>	307±26*	408±3*	429±21*	10.8±5.6 †	14.8±2.0 †	13.5±4.6 †	3.25±1.9

Values are mean (N=8) ± SD. BW: \* indicates D, DX had significantly lower weights than C,CX (p<0.05) BG:† indicates D,DX post-diabetes induction, had significantly higher BG than C,CX (p<0.05) Insulin Dose: non-significance p=0.061

Table 2.3 *Temperature Measures in Exercised Groups*

Groups	Midpoint		Endpoint	
	Pre	Post	Pre	Post
<b>Control Exercised (CX)</b>	38.6±0.4	40.1±0.42*	40.6±0.6	42.0±0.3*
<b>Diabetic Exercised (DX)</b>	35.5±0.8	36.2±1.25	39.3±1.2	41.1±1.0
*indicates post-measure is significantly different from pre-measure p<0.001				

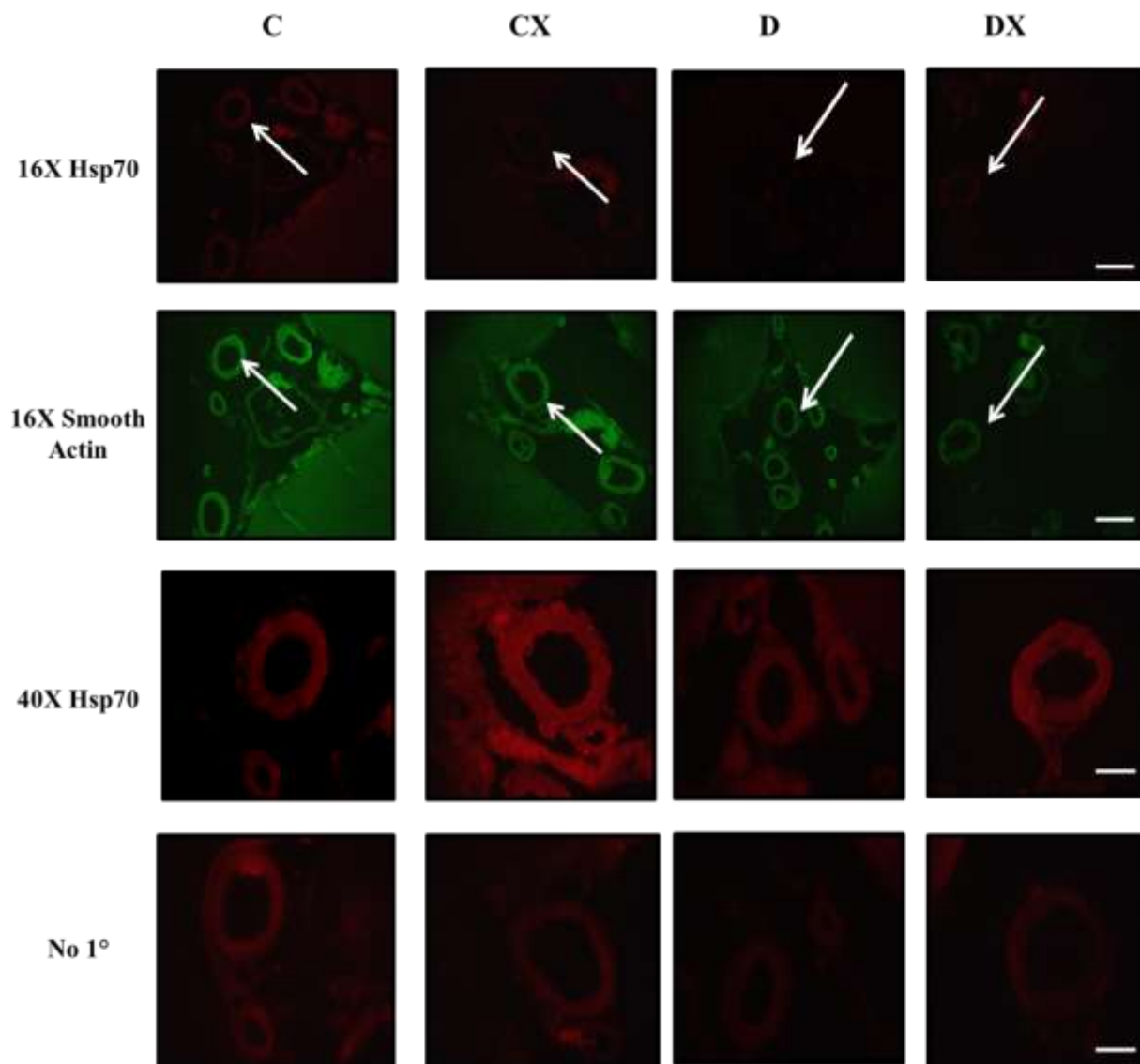
**Plate 1.** Visualization of Large cerebral vessels and Hsp70 protein signal in cerebral cortex across Control (C), Control Exercise (CX), Diabetic Sedentary (D) and Diabetic Exercised (DX) groups.

First two rows illustrate cerebral vessels in the cortex and smooth muscle cells. Bottom row indicates negative control to which no primary antibody was applied during the immunohistochemistry protocol.

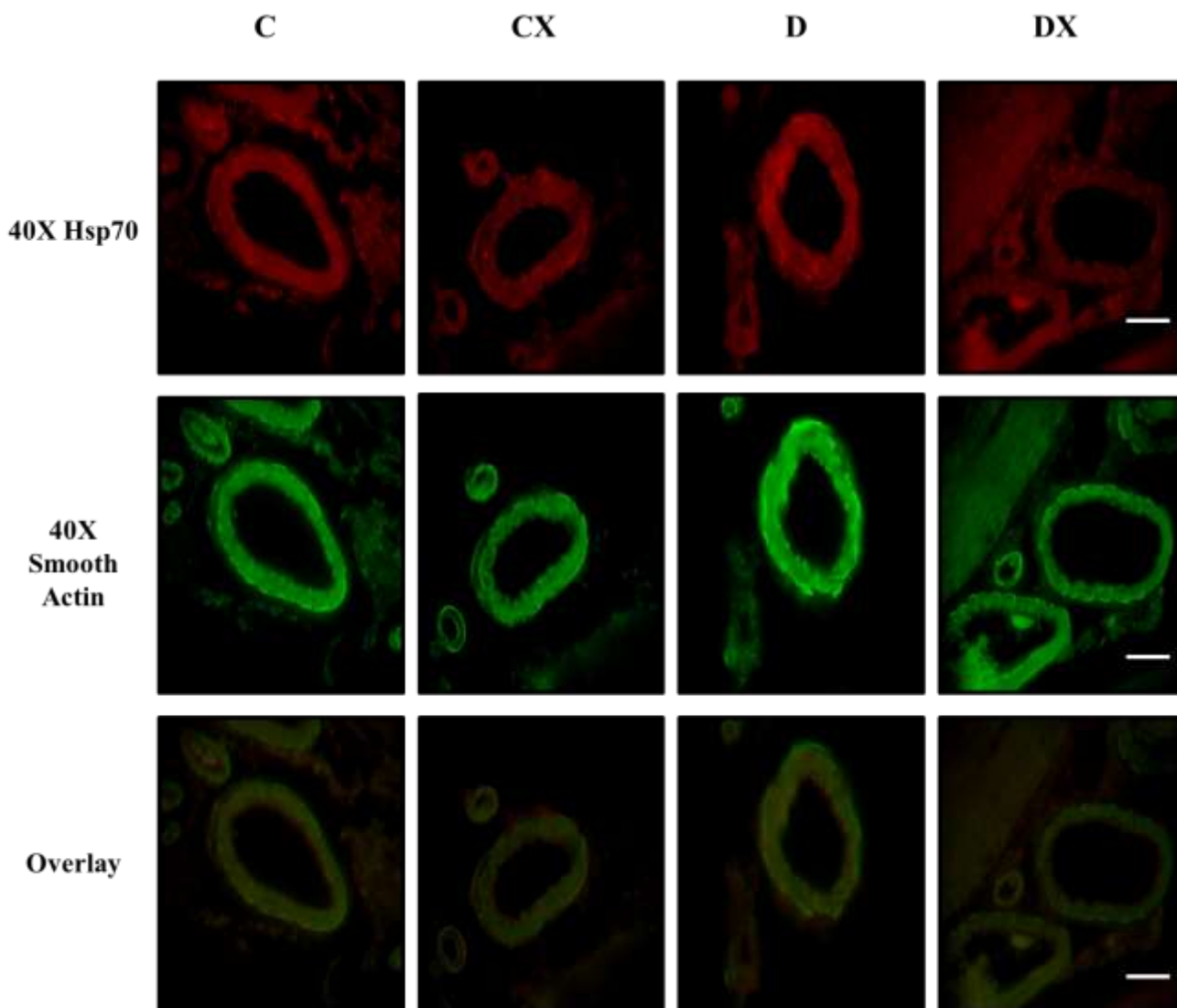
White arrows identify the vessel of interest at (16X) to its (40X) magnification.

Scale bar 16X=0.5mm

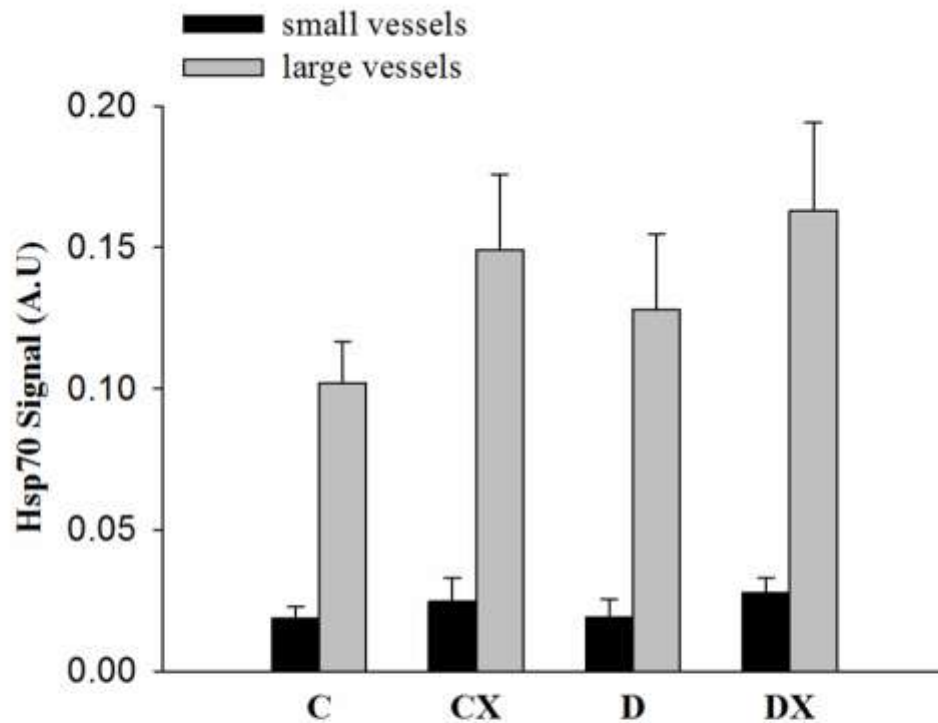
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**Plate 2.** Co-Localization of Hsp70 (red) and smooth muscle cells (green) in large cerebral blood vessels (40X) across control sedentary (C), control exercised (CX), diabetic sedentary (D) and diabetic exercised (DX) groups. Third row overlay of protein to smooth muscle cells, illustrates Hsp70 signal density is primarily localized to the smooth muscle area.  
Scale bar 40X= 0.05mm



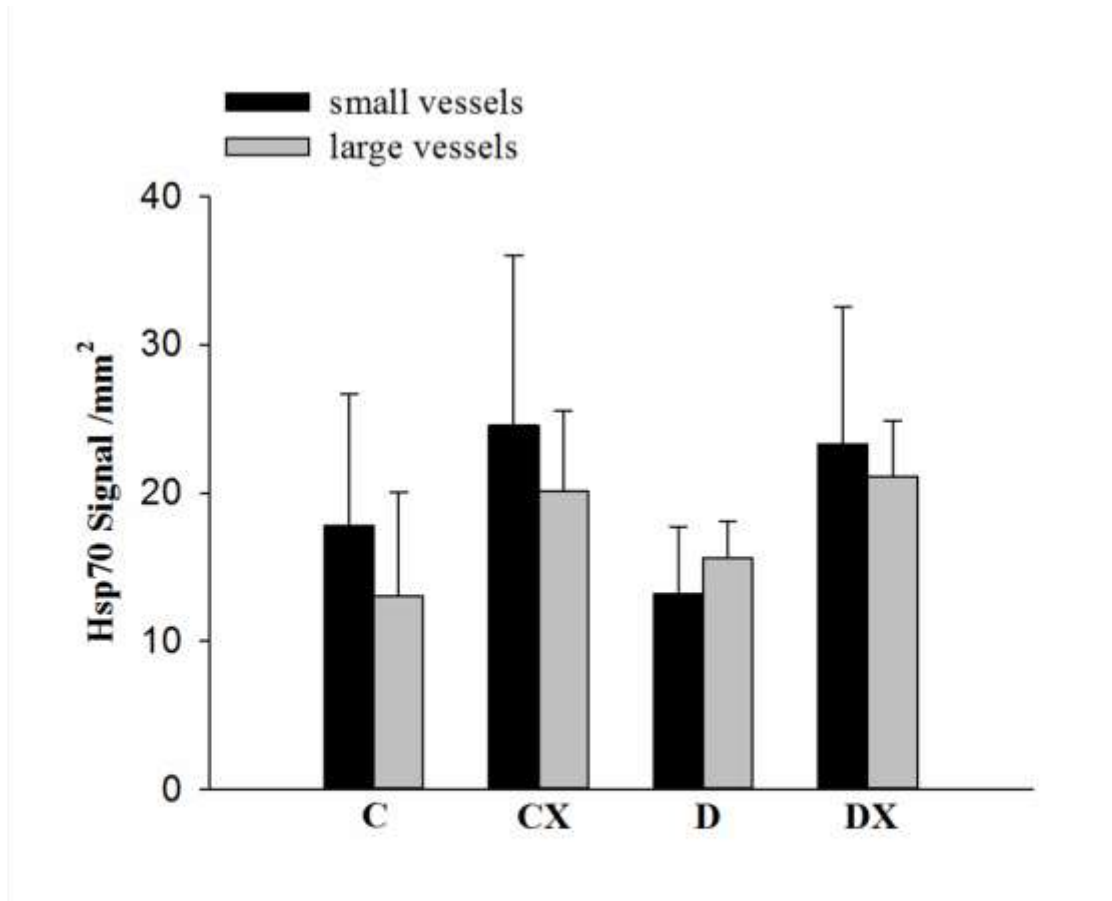




**Fig 2.2 Hsp70 Protein Signal Expression in Small and Large Cerebral Blood Vessels.**

Protein signal content expressed as mean  $\pm$ SD.

In small and large vessels, there is a main effect of training on Hsp70 expression ( $p < 0.05$ ). The signal content was greater in larger vessels when compared to small vessels.



**Fig 2.3 Hsp70 Expression adjusted for SMA in Small and Large Cerebral Blood Vessels.**

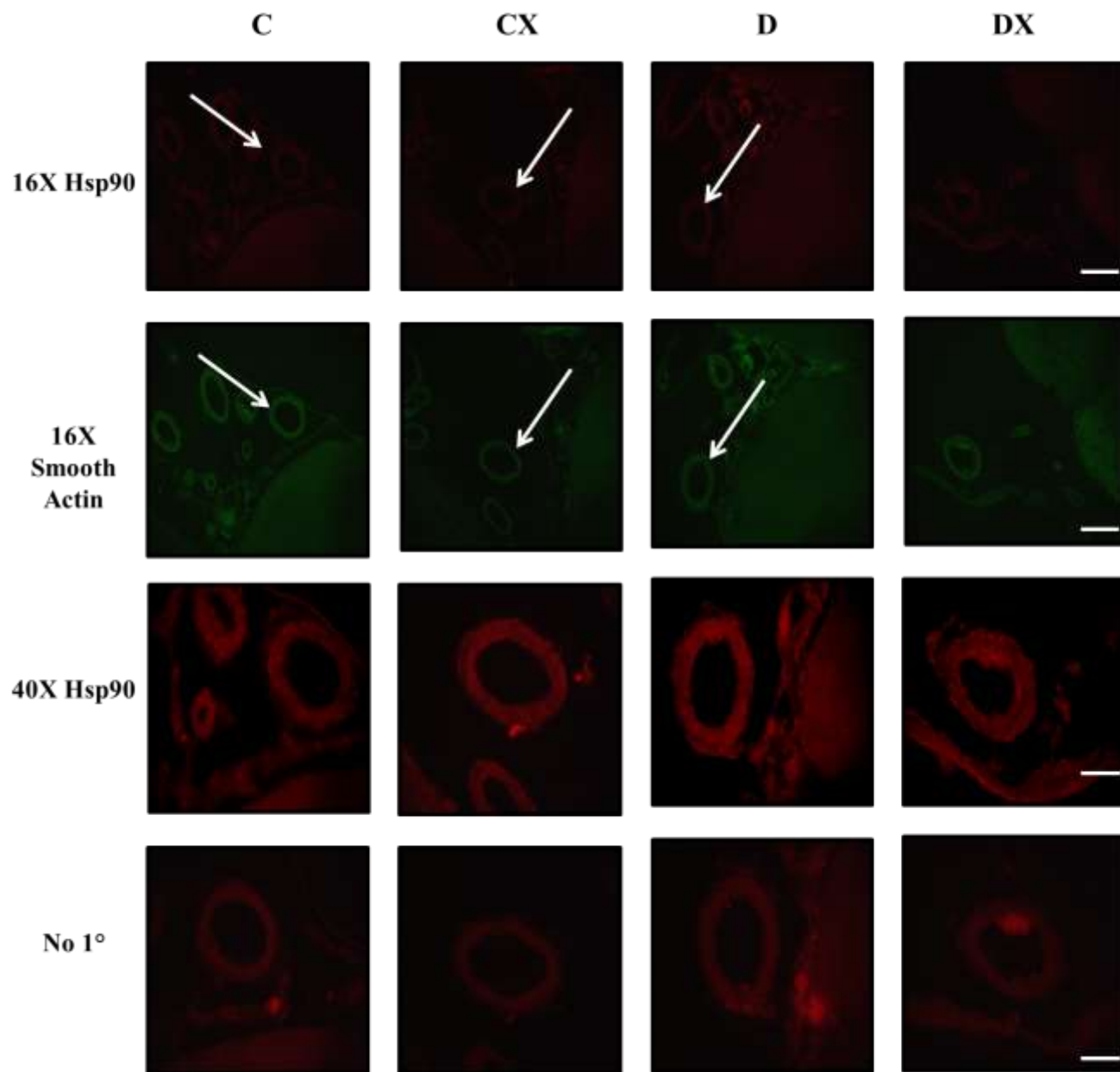
Protein signal density over SMA expressed as mean  $\pm$ SD.

When adjusted for SMA, there is a main effect of training on Hsp70 expression ( $p < 0.001$ ).

**Plate 3.** Visualization of Large cerebral vessels and Hsp90 protein signal in cerebral cortex across Control (C), Control Exercise (CX), Diabetic Sedentary (D) and Diabetic Exercised (DX) groups. First two rows illustrate cerebral vessels in the cortex and smooth muscle cells. Bottom row indicates negative control to which no primary antibody was applied during the immunohistochemistry protocol. White arrows identify the vessel of interest at (16X) to its (40X) magnification.

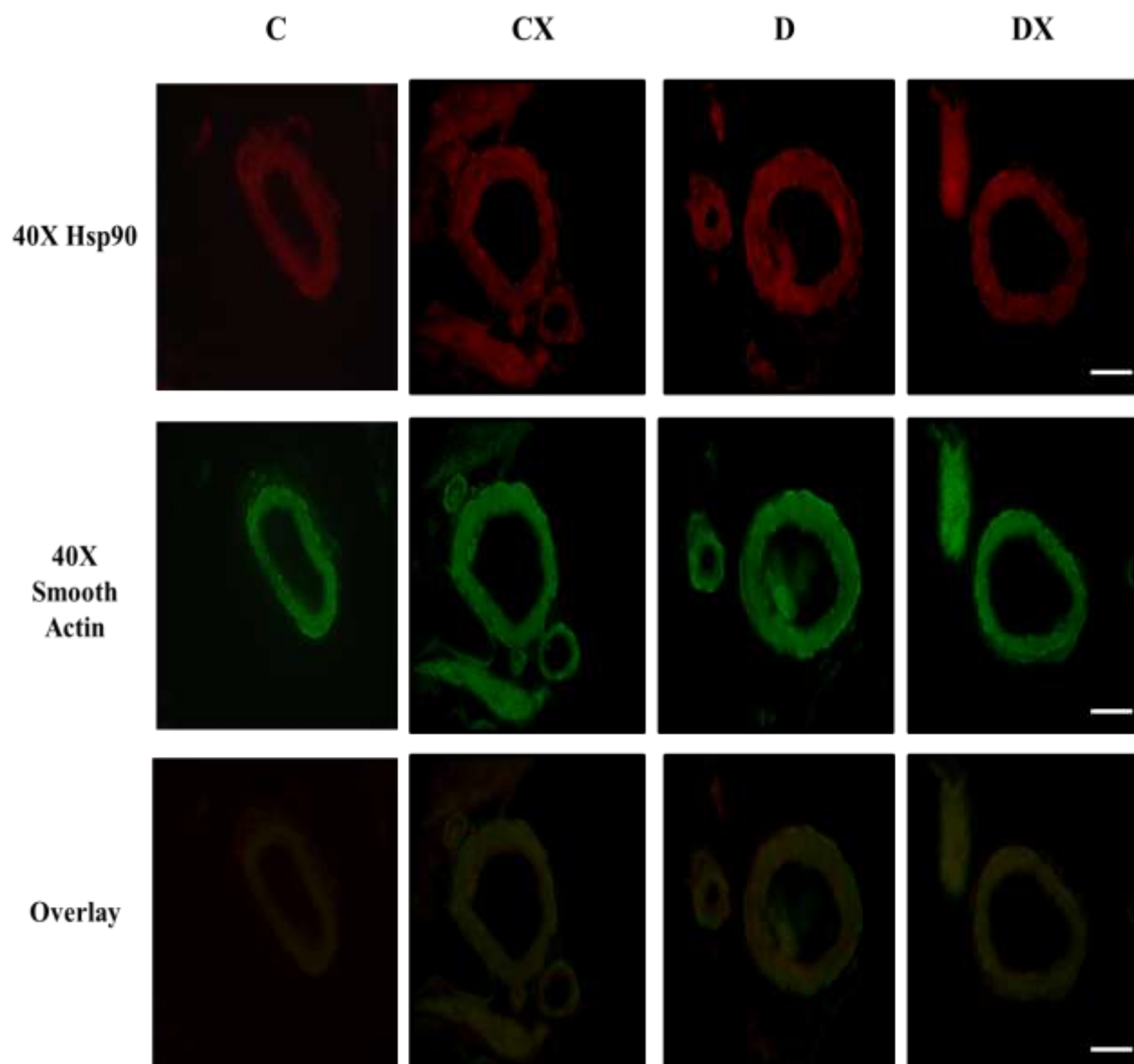
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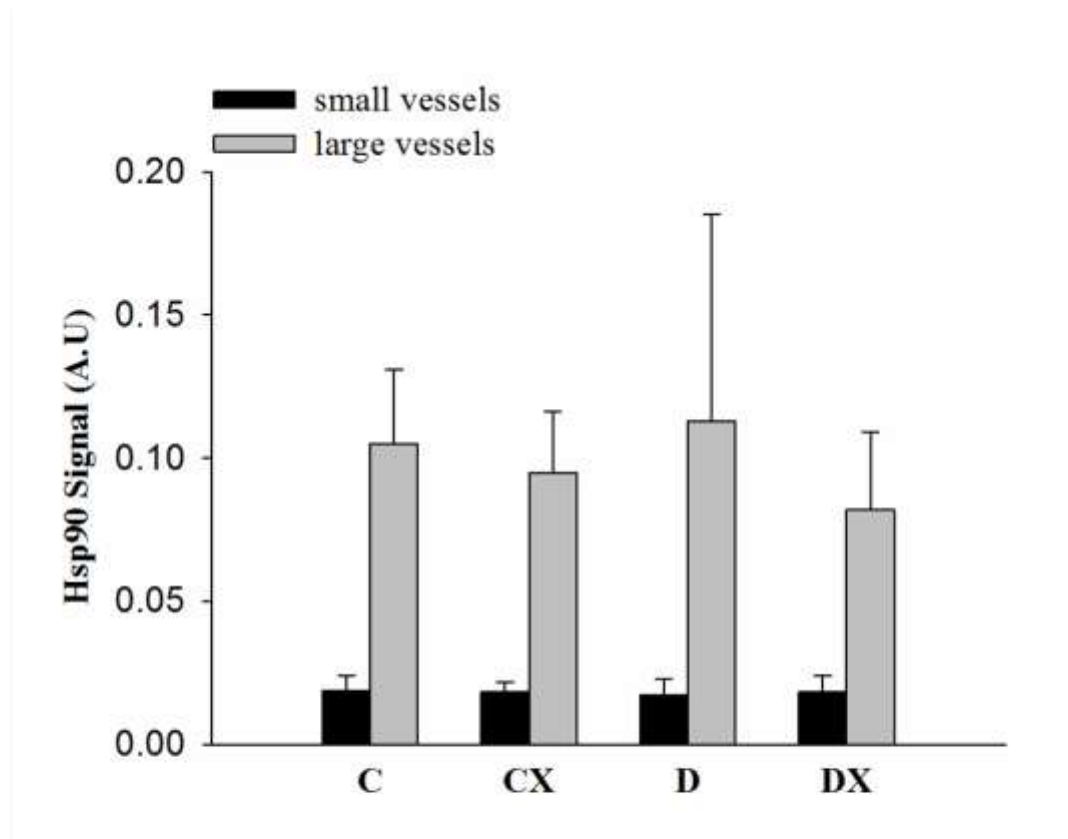
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**Plate 4.** Co-Localization of Hsp90 (red) and smooth muscle cells (green) in large cerebral blood vessels (40X) across control sedentary (C), control exercised (CX), diabetic sedentary (D) and diabetic exercised (DX). Third row overlay of protein to smooth muscle cells, illustrates Hsp90 signal density is primarily localized to the smooth muscle area.

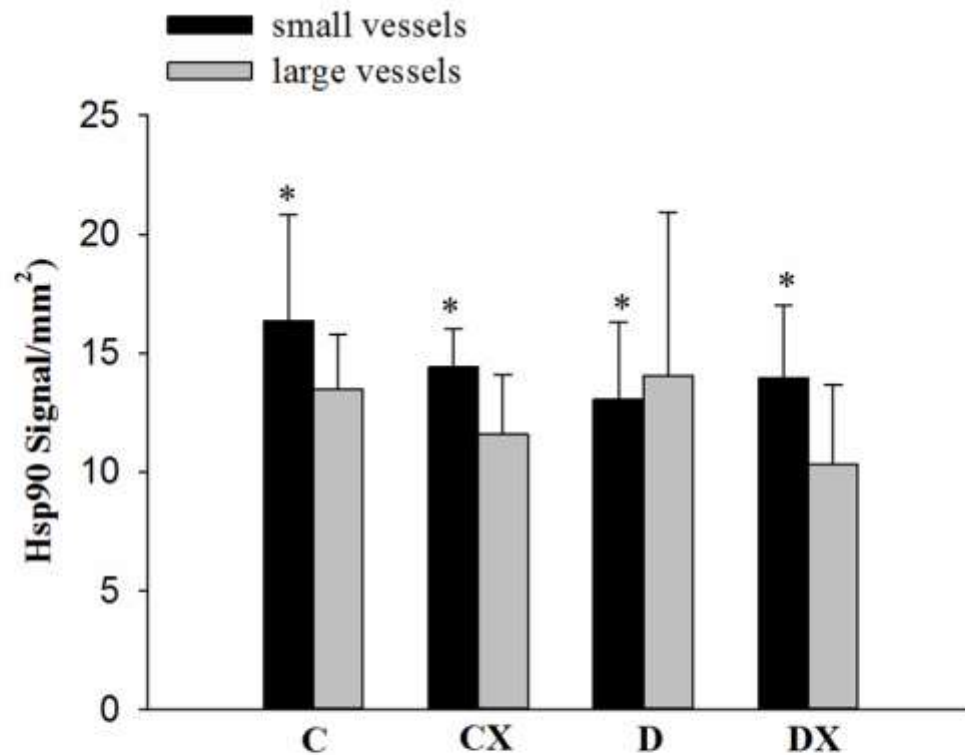
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**Fig 2.4 Hsp90 Protein Signal Expression in Small and Large Cerebral Blood Vessels.** Protein signal content expressed as mean  $\pm$ SD.

In small and large vessels, there is no main effect of training on Hsp90 expression ( $p < 0.05$ ). The signal content was greater in larger vessels when compared to small vessels.



**Fig 2.5 Hsp90 Expression adjusted for SMA in Small and Large Cerebral Blood Vessels.**

Protein signal density over SMA expressed as mean  $\pm$ SD.

When adjusted for SMA, (\*) indicates there is a main effect of vessel size ( $p < 0.05$ ).

There is still no main effect of training on Hsp90 expression ( $p > 0.05$ ).



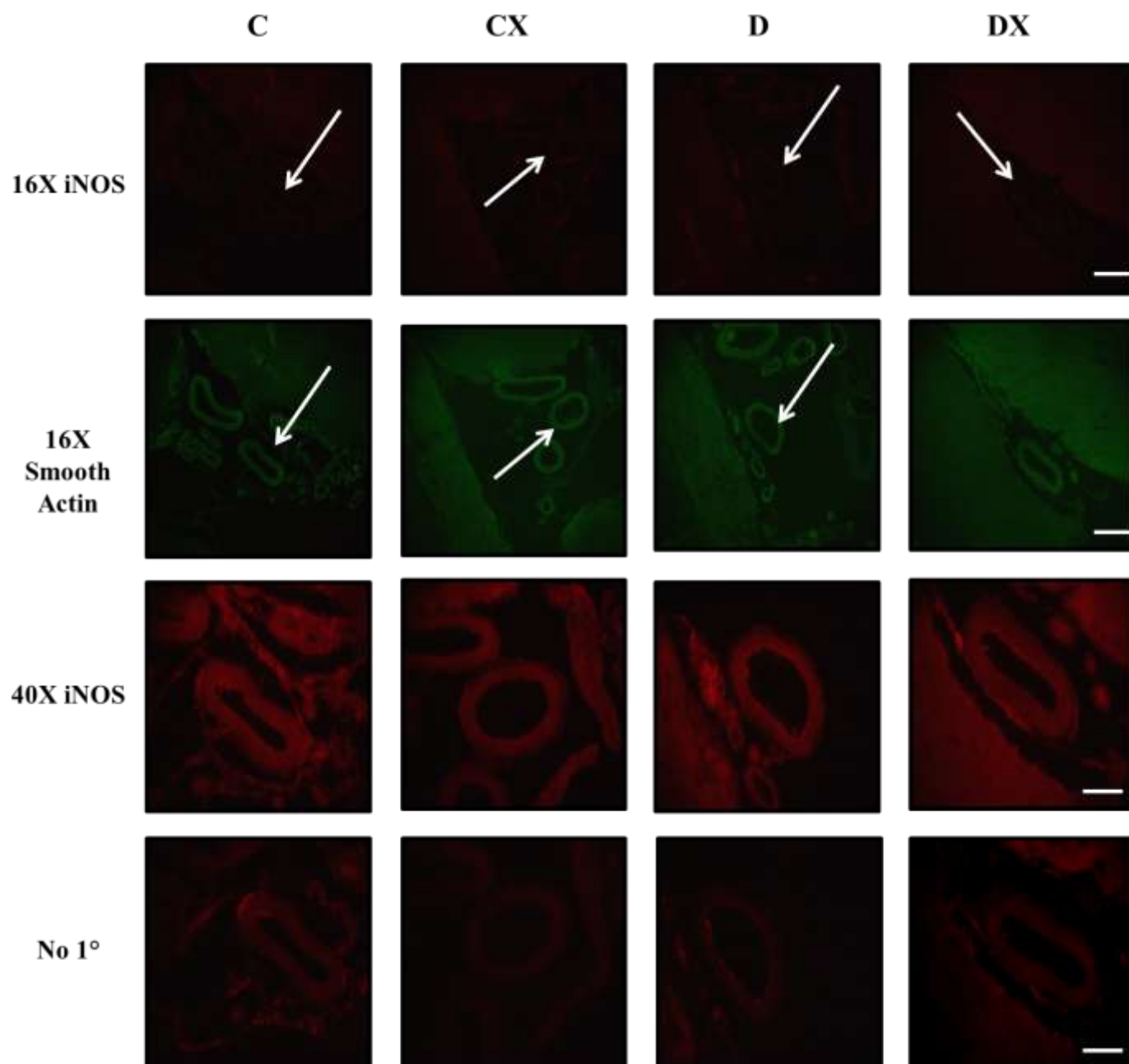
**Plate 5.** Visualization of Large cerebral vessels and iNOS protein signal in cerebral cortex across Control (C), Control Exercise (CX), Diabetic Sedentary (D) and Diabetic Exercised (DX) groups.

First two rows illustrate cerebral vessels in the cortex and smooth muscle cells. Bottom row indicates negative control to which no primary antibody was applied during the immunohistochemistry protocol.

White arrows identify the vessel of interest at (16X) to its (40X) magnification.

Scale bar 16X=0.5mm

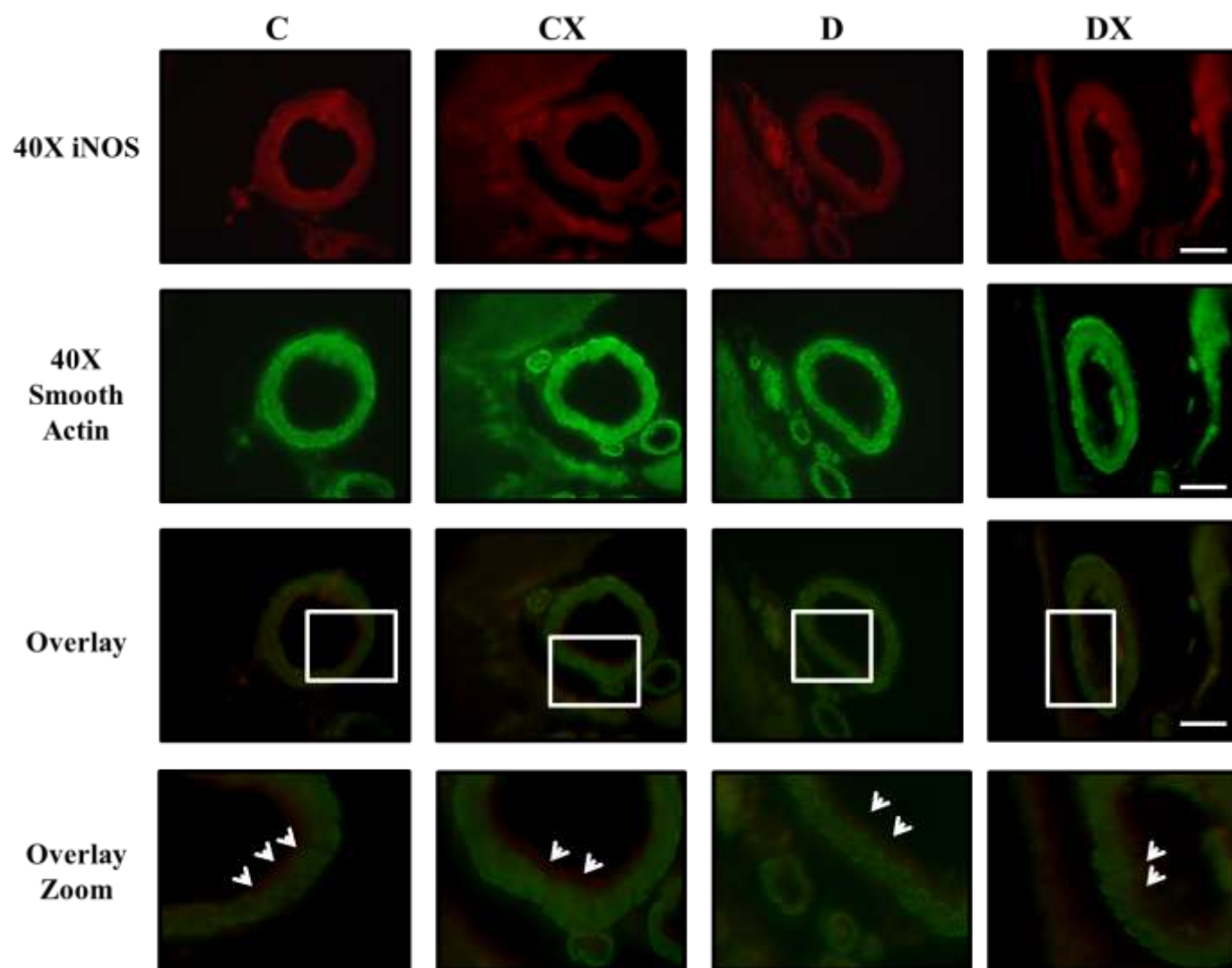
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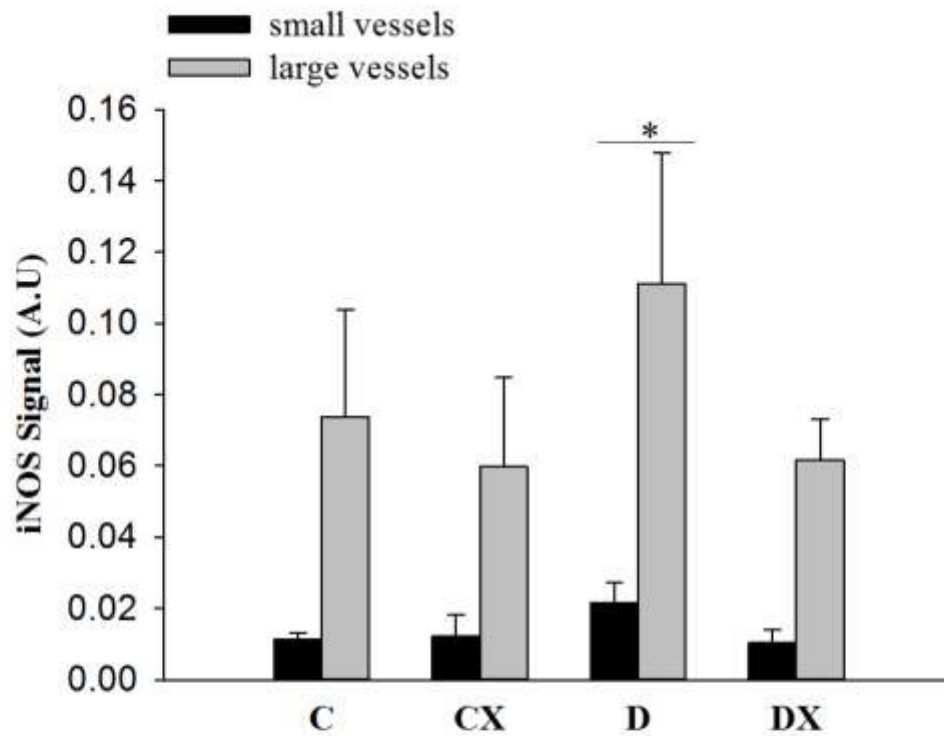


**Plate 6.** Co-Localization of iNOS (red) and smooth muscle cells (green) in large cerebral blood vessels (40X) across control sedentary (C), control exercised (CX), diabetic sedentary (D) and diabetic exercised (DX). Third row overlay of protein to smooth muscle cells illustrates though iNOS signal density is primarily localized to the smooth muscle area.

Boxed areas correlate to the overlay zoom and the white arrowheads point to the potential endothelial localization of iNOS.

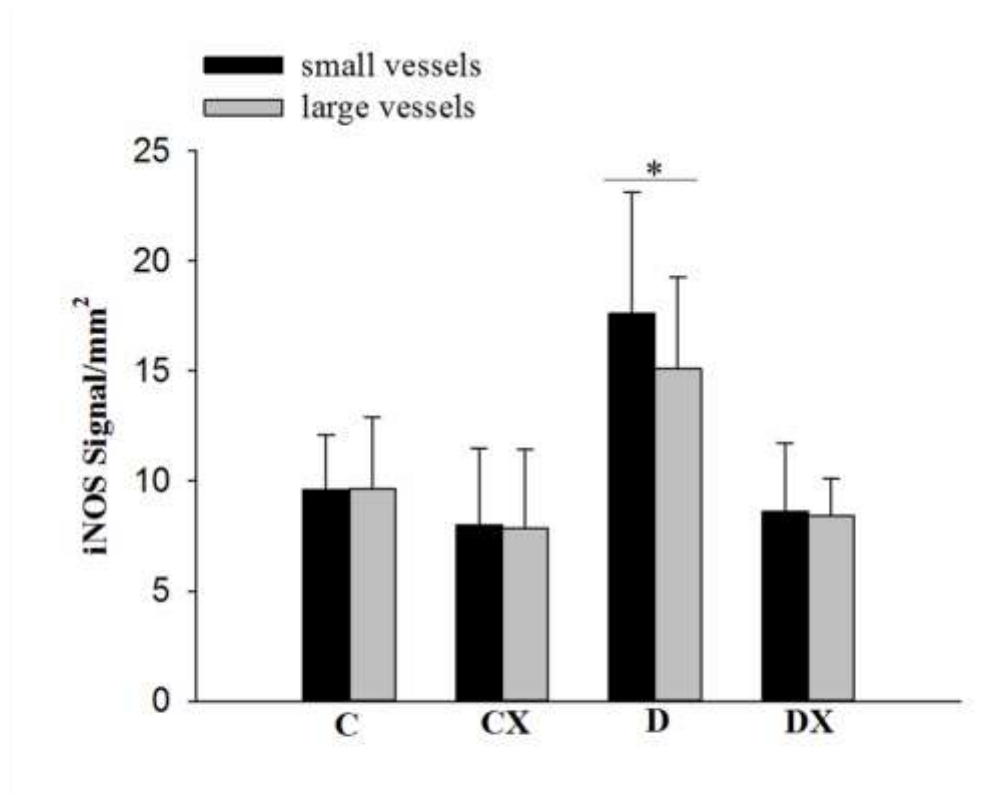
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**Fig 2.6 iNOS Protein Signal Expression in Small and Large Cerebral Blood Vessels.** Protein signal content expressed as mean  $\pm$ SD.

(\*) Indicates in both small and large vessels, there was a main effect of disease and training on iNOS expression ( $p < 0.05$ ). There was a main effect of disease and training on iNOS expression. The signal content was greater in larger vessels when compared to small vessels.



**Fig 2.7 iNOS Expression adjusted for SMA in Small and Large Cerebral Blood Vessels.**

Protein signal density over SMA expressed as mean  $\pm$ SD.

When adjusted for SMA, (\*) indicates there is a main effect of disease on ( $p < 0.001$ ) and effect of training ( $p < 0.001$ ) on iNOS expression. .

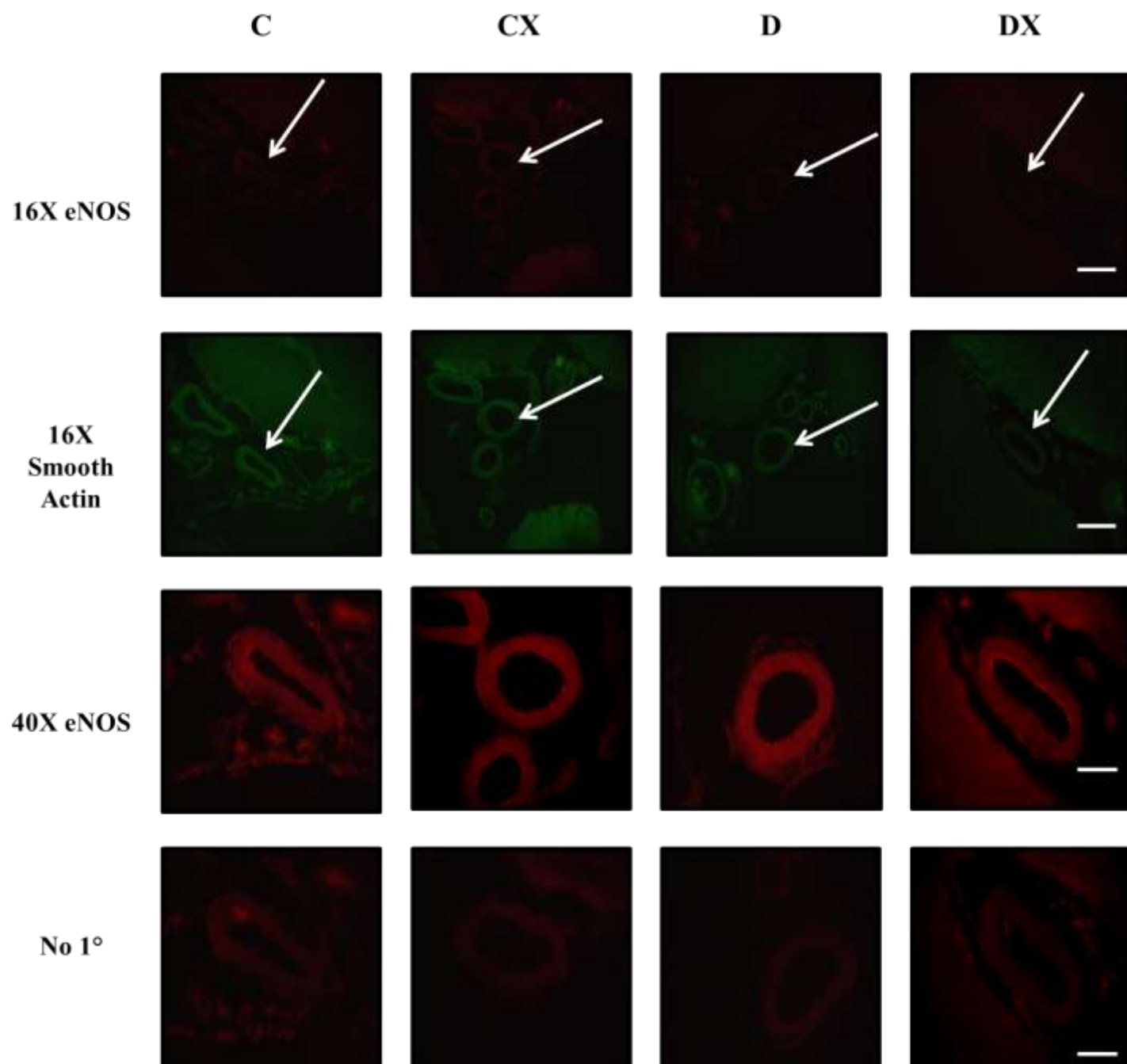
**Plate 7.** Visualization of Large cerebral vessels and eNOS protein signal in cerebral cortex across Control (C), Control Exercise (CX), Diabetic Sedentary (D) and Diabetic Exercised (DX) groups.

First two rows illustrate cerebral vessels in the cortex and smooth muscle cells. Bottom row indicates negative control to which no primary antibody was applied during the immunohistochemistry protocol.

White arrows identify the vessel of interest at (16X) to its (40X) magnification.

Scale bar 16X=0.5mm

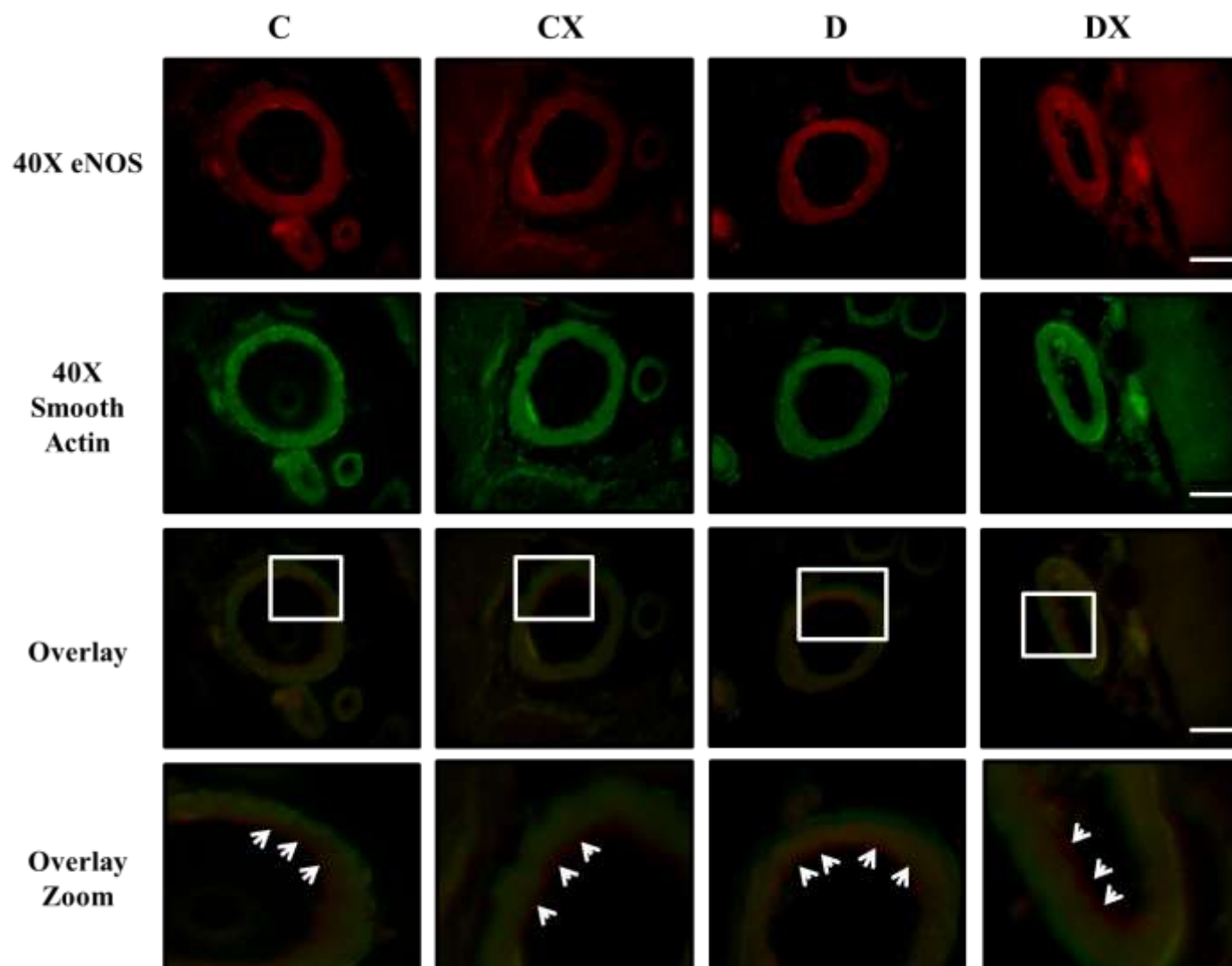
Scale bar 40X=0.05mm

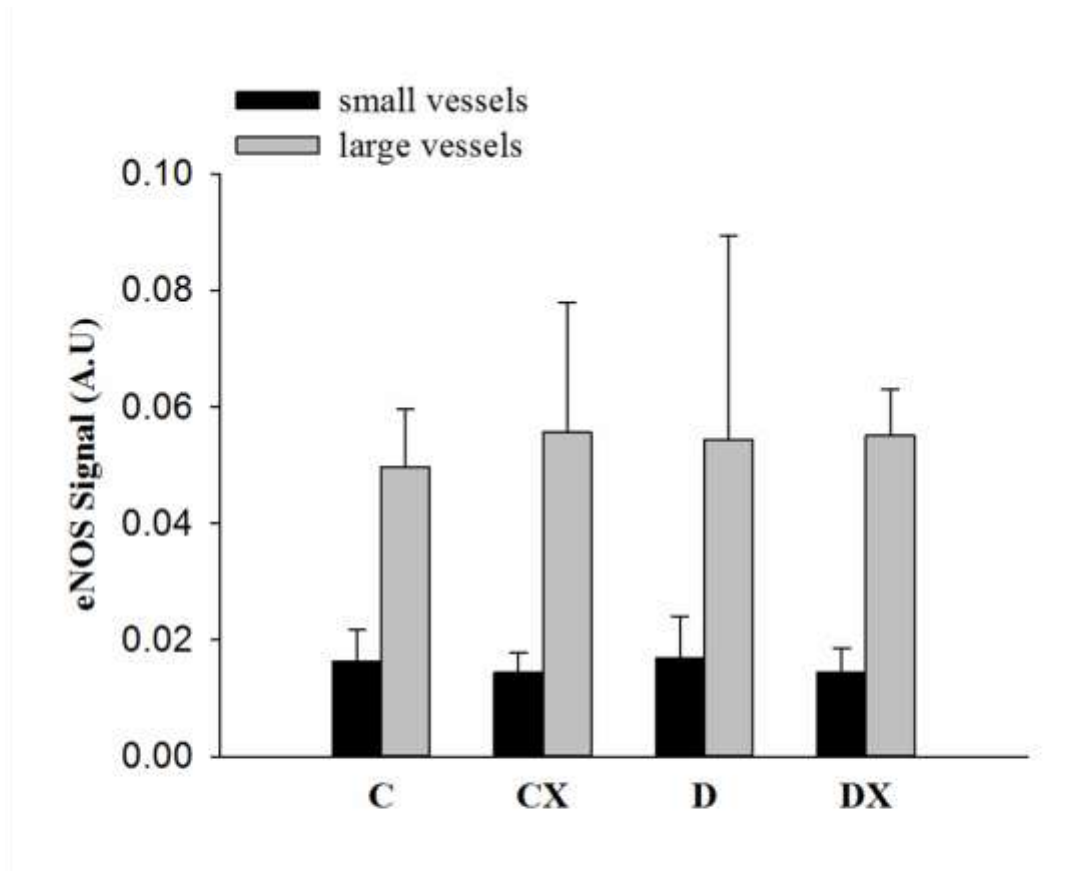




**Plate 8.** Co-Localization of eNOS (red) and smooth muscle cells (green) in large cerebral blood vessels (40X) across control sedentary (C), control exercised (CX), diabetic sedentary (D) and diabetic exercised (DX). Third row overlay of protein to smooth muscle cells illustrates though eNOS signal density is localized to the smooth muscle area. Boxed areas correlate to the overlay zoom and the white arrowheads point to the potential endothelial localization of eNOS.

Scale bar 40X= 0.05mm

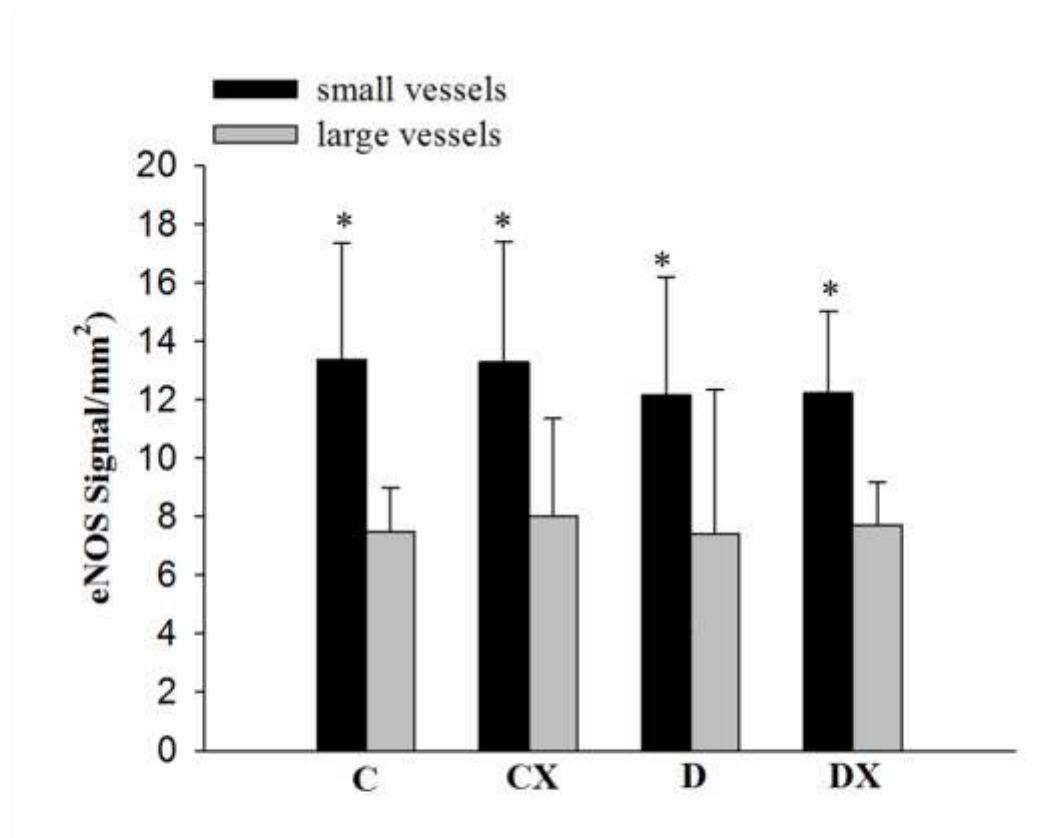




**Fig 2.8 eNOS Protein Signal Expression in Small and Large Cerebral Blood Vessels.**

Protein signal content expressed as mean  $\pm$ SD.

In small and large vessels, there is no main effect of training on eNOS expression ( $p < 0.05$ ). The signal content was greater in larger vessels when compared to small vessels.

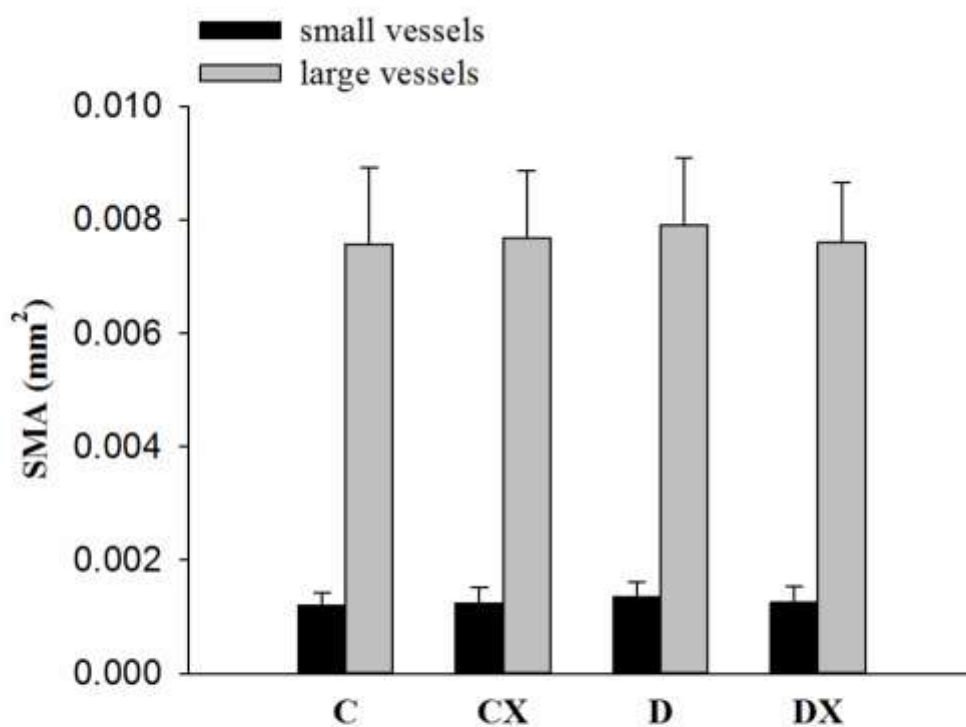


**Fig 2.9 eNOS Expression adjusted for SMA in Small and Large Cerebral Blood Vessels.**

Protein signal density over SMA expressed as mean  $\pm$ SD.

When adjusted for SMA, (\*) indicates there is a main effect of vessel size ( $p < 0.001$ ).

There is still no main effect of training on eNOS expression ( $p > 0.05$ ).



**Fig 2.10 Smooth Muscle Area (SMA) in Small and Large Cerebral Blood Vessels.** There was no significant difference in SMA between all groups, in both small and large vessels ( $p>0.05$ ).

## 2.4 Discussion

Ischemic stroke is often a result of endothelial dysfunction and accelerated atherosclerosis, conditions attributed to prolonged hyperglycemic exposure exhibited in T1DM (21). Aerobic exercise training has been proven to elicit adaptive neuroprotection, particularly through reduced brain injury, demonstrated in *in vivo* models of ischemic stroke (12, 14). A heat shock response (HSR)-induced, anti-inflammatory mechanism has been suggested as an underlying this neuroprotection (8, 9). Nevertheless, the biological underpinnings of aerobic exercise training and HSP-mediated effect on the cerebrovasculature are not well known, especially in chronic disease conditions such as T1DM. Hence the purpose of this study was to evaluate the effect of 10-weeks of high intensity aerobic training in a slight hyperglycemic T1DM condition, on vascular HSP and NOS expression. The working hypothesis fittingly afforded the morphological localization of these proteins in cerebral vessels following exercise.

Fluorescent co-localization of protein overlaid with VSMCs illustrates that the Hsp70, Hsp90, iNOS and eNOS expression is primarily localized to the SMA. These observations are similar to those of Kim et.al (26), whereby heat shock stress induced Hsp70 expression in mice aortic vascular smooth muscle cells. Through co-association and localization of Hsp90 in the mesenteric tissue, Sessa et.al (40,41) determined Hsp90 is primarily localized to vascular smooth muscle cells as well (46, 47). The present study suggests that the vascular localization of HSPs in cerebral vessels is similar to those in the systemic vasculature. For iNOS and particularly eNOS signal content, though the signal is primarily localized to SMA there may be a tendency towards vascular endothelial expression though this study does not confirm this assumption. The

observations of this study are in line with a study by Sun et al. (43) which evaluated the effects of different exercise modalities on vascular localization of eNOS and iNOS in the murine aorta (50). Their study confirmed endothelial and primarily perivascular (surrounding smooth muscle) localization of eNOS and iNOS in endurance trained rat aortic sections.

At a first glance, these results illustrate the capacity for cerebral blood vessels to induce a HSR. This is the first study to examine the expression and localization of HSPs in cerebral vessels. Previous studies have demonstrated Hsp70 induction in brain tissues with chronic exercise training but this is the first study to demonstrate a cerebrovascular localization of Hsp70 following aerobic high intensity training (3,27). Ten-week, high intensity training alone (CX) and in T1DM rats (DX), induced a significant Hsp70 expression in both small and large vessels when compared to their respective non-trained groups(C, D). The training induced Hsp70 expression was not blunted in the controlled slight hyperglycemic, insulin treated T1DM model that we employed. These results are in contrast to a previous rat diabetic model that illustrated a blunted training effect on whole brain Hsp70 protein content (28). However, it should be noted that the previous study sustained diabetes at a higher grade of hyperglycemia (>20mmol/L), in the absence of exogenous insulin when compared to slight hyperglycemic model used in the present study (9-15mmol/L). The presence of insulin appears to greatly affect the response of the animals for a variety of measures (34). For instance, Li.et al. (24) have illustrated an insulin mediated induction of Hsp70 expression in myocardial tissue. Thus, the Hsp70 expression observed in trained diabetic animals could be a synergistic effect of lower

grade hyperglycemia maintained in this study and insulin mediated low-level induction of Hsp70.

In the present study, both Hsp90 and eNOS expression remained constant in trained (CX, DX) and untrained (C, D) groups, across both small and large cerebral vessels. This observation is interesting in that, prior investigations have illustrated an increase in skeletal muscle Hsp90 and eNOS protein content to facilitate an increase in blood flow to working muscle (4,7). Yamaguchi et al. also noted that whole brain Hsp90 content remained unchanged in from the control to the diabetic state however there is no clear interpretation of this result (44). To our knowledge, this study is the first to investigate the vascular Hsp90 expression in the brain from exercise trained animals. The brain is a unique organ that tightly regulates its vasodilatory mechanisms which may in part explain the unaltered expression of Hsp90 across varying conditions. In the normotensive state, Hsp90 is associated with eNOS, and upon stimulation by various factors, including exercise, the association between the two proteins increases, resulting in baseline NO production which in turn, promotes vasodilation (15, 51). In the brain, CBF is tightly autoregulated, unlike in skeletal muscle where there are large variations in blood flow that occur in response to exercise (40, 42). Moderate intensity training, 60% VO<sub>2</sub>max, induces a marked elevation in global CBF (gCBF) during exercise. In contrast, with high intensity training (VO<sub>2</sub>max >60%), there is a significant decline in gCBF to baseline level during exercise, despite a significant increase in metabolic demand. It is then postulated that short-term hyperventilation sustains cerebral function under these circumstances (42). It can then be suggested that in our study with higher intensity exercise in the brain, there were no significant differences in vascular Hsp90 and eNOS



protein content across all groups (C,CX,D,DX) conceivably due to an intrinsically tight autoregulation that strives to keep vasodilatory mechanisms as constant as possible.

In this study the cerebrovascular iNOS protein content in sedentary diabetic animals (D) was significantly greater than all other groups, in both small and large vessels. These data agree with studies that have illustrated cerebrovascular iNOS up regulation during pro-inflammatory events that include cerebral ischemia, and this up regulation is site specific to the cortical region of ischemia in acutely stressed rats (33, 57). Since it has been reported (33) that iNOS is not present in the CNS under normal conditions, this observation identifies a propensity towards cerebrovascular inflammation even in moderate insulin treated hyperglycemic conditions. The most intriguing result is the normalization of vascular iNOS content observed in trained T1DM animals (DX) when compared to sedentary T1DM (D).

Exercise stress induces an HSR which in turn helps initiate an anti-inflammatory milieu (3, 17). It has been postulated that upstream overexpression of Hsp70 imposes downstream inhibition of iNOS and prevents the subsequent accumulation of high iNOS protein levels in various organ tissue lysates including brain (24, 25). A proposed underlying mechanism to this Hsp70 prompted protection is Hsp70 inhibition of NF- $\kappa$ B via of its interaction with the NF- $\kappa$ B inhibitor I- $\kappa$ B.

With specific reference to eNOS, in this study there were no significant differences in eNOS protein content between groups, particularly between T1DM and non-T1DM groups. In contrast to prior studies illustrating a significant decrease in eNOS bioactivity and accumulation in VSMCs, this study observed a minute overall decrease in

the T1DM groups when compared to sedentary and trained non-T1DM groups but these data were non-significant. One possible explanation could be the exogenous insulin and attenuated degree of hyperglycemia ( $<20\text{mmol}$ ) in this study, wherein the previous studies by Mohan et,al (37) maintained higher grade or ‘severe’ hyperglycemia ( $>25\text{mmol}$ ). The collective data from iNOS and eNOS in this model of T1DM depicts a story whereby the progression of vascular inflammation precedes the phenotypic onset of endothelial dysfunction.

The midpoint and endpoint temperature data in brief illustrate a non-significant increase from pre to post temperatures in diabetic trained animals (DX) when compared to significant increases from pre to post observed in trained animals (CX). In addition, diabetic animals experienced higher pre exercise temperature measures when compared to their non-diabetic trained counterparts. This observation in part could explain for the HSP expression observed in diabetic animals in this study and could also be a factor of disease induced symptoms that distort thermoregulatory mechanisms. This speculative distortion could then have various downstream impacts on HSP expression however, the exact mechanisms are not clearly understood at this point.

The vascular signal intensity was adjusted for SMA in small and large cerebral vessels, in order to determine if and any potential impact of vessel size existed on the individual protein expression. The SMA alone did not change between the four groups in small or large vessels, with factors of disease (D) and training interventions (DX, CX) when compared to sedentary (C). We had examined this because previous data suggested that diabetes was associated with vascular hypertrophy (4, 52). The fact that we did not observe any change in SMA in the present investigation likely underscores the fact that

we were using a controlled low-grade hyperglycemia diabetic model rather than an untreated diabetic animal. The short duration of the study, given that vascular hypertrophy manifests at a more progressive stage of T1DM may have also been a factor. Though the pattern of expression was similar in small and large vessels across all four proteins of interest, when adjusted for SMA, Hsp90 and eNOS signal content had a main effect of size. The Hsp90 and eNOS protein content was significantly greater in smaller vessels when compared to larger vessels, prominent more so with eNOS expression. These observations are similar to recently published data from our lab, where Murias et.al (32) demonstrated greater eNOS protein content in smaller vessels, femoral arteries, when compared to large branches of vessels such as the iliac artery and the descending branch of aorta from in trained and untrained diabetic rats (38). In that study, a greater concentration of eNOS protein in smaller vessels was implicated in the faster rate of vasorelaxation observed in femoral when compared to the larger aorta and iliac. Hsp90 signal density was greater in smaller vessels than larger vessels, and the size effect was significant, indicating that Hsp90 maintains a pattern of bioactivity parallel to that of eNOS in the cerebrovasculature. In addition, the SMA adjustments denote the importance of quantifying the signal content to area in order to gage a more normalized expression factoring in the size.

## **2.5 Conclusion**

Until now, no observations exist on cerebrovascular HSP and NOS expression in a controlled low-grade hyperglycemic, insulin treated diabetes model and especially that, with chronic exercise training intervention. The objective of this study was to evaluate the effect of 10-weeks of high intensity aerobic training alone and in a slightly

hyperglycemic T1DM condition, on HSP and NOS expression and concomitant localization in cerebral vessels. Although animal models have frequently been employed to study the effect of exercise on T1DM, the severity of hyperglycemia in those particular models have imposed high levels of physiological stress such that, the results are difficult to translate (7). In fact, blood glucose levels in these animals have been well above the normal limits observed in human patients. In the present study, we attempted to mimic a low level of hyperglycaemia often found in humans, even in the presence of insulin supplementation (34). Hence this study has more translational relevance.

The results supported the hypothesis that a 10-week high intensity training protocol would be associated with a significant increase in Hsp70 in control exercised animals (CX); however, this response was not attenuated or blunted as expected in the exercise trained diabetic group (DX). Interestingly, 10-week training protocol in the diabetic group significantly blunted pro-inflammatory iNOS protein content when compared to the increase in the pro-inflammatory iNOS observed in diabetic sedentary group (D). This suggests that although many vascular properties, such as SMA, may be well maintained when insulin is present and hyperglycemia is moderate, markers of chronic cerebrovascular inflammation are still present. Importantly, exercise training appears to mitigate this response even in the presence of reduced circulating insulin and unaltered blood glucose levels. It is likely that elevations in vascular Hsp70 subsequent to exercise training may protect against the progression of atherosclerosis and potentially stroke by inhibiting iNOS and subsequent vascular inflammation (17, 54). This warrants further study into the direct interactions between Hsp70 and pro-inflammatory regulators, in order to address and advance towards potential therapeutic strategies.

The results for Hsp90 and eNOS are in not in concurrence with our hypothesis, in that there were no significant differences in vascular Hsp90 and eNOS protein content amongst groups. We speculate that this can be attributed to an intrinsically tight cerebral autoregulation that strives to keep vasodilatory mechanisms stable in addition to the model of T1DM implemented in this translational approach. The outcomes of this study have touched upon the HSP expression underlying exercise induced vascular protection and merits future investigations in HSR regulation that can be translated into interventions in chronic diseases that include components of cerebral vascular pathology.

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## CHAPTER 3

### 3.1 Limitations and Assumptions

This study sought to investigate a unique concept of cerebrovascular HSP and NOS expression, whereby the novelty of the subject made it difficult to interpret all results with complete assurance. This is mostly due to the lack of previous studies implementing a more physiological T1DM model, such as this study whereby exogenous insulin therapy afforded a more controlled low-grade hyperglycemia. In addition, there exists a lack of interventions examining chronic high intensity exercise training alone and in a T1DM model, on cerebrovascular HSP and NOS expression.

Having said that, there are considerations that could reinforce the results and these considerations include directed characterization of molecular regulators and client proteins of the HSPs studied. In particular, the speculative discussion around Hsp70 and iNOS could be clarified with characterization of intermediate regulators, including NF- $\kappa$ B, I- $\kappa$ B and TNF $\alpha$ . A closer look at the molecular interactions, perhaps *in situ hybridization* or *immunoprecipitation assay* protocols could highlight more precise interactions between Hsp70 and the aforementioned pro-inflammatory regulators. In effect these assays would bolster the speculations about the anti-inflammatory role of Hsp70 interactions with downstream factors, including in this study iNOS. By extension, evaluating direct interactions between Hsp90 and its clients, including eNOS, IKK $\beta$  by means of *immunoprecipitation* assays could highlight how this housekeeping protein regulates between disease and non-disease states.

Recent applications of in vivo research has shifted towards genetic manipulations that target studying specific proteins of interest through transgenic knock out and knock down models. The transgenic knockout/knockdown or overexpression of (Hsp70+/-), (Hsp70 -/-) in recent studies have elucidated many regulatory functions of Hsp70 and future studies could potentially investigate and extract the precise role of Hsp70 in this model and clarify if Hsp70 is a primary player in the observed neuroprotection.

The experimental results are speculations that could be bolstered with cerebral blood flow (CBF) measures which were not taken into account for the experiment design in this study. CBF measures could potentially shed light on the functional changes, particular following exercise training and the response to lower grade hyperglycemia in the cerebrovascular setting. CBF data would contribute to elucidating the flow mediated influence on vascular shifts that would better explain the observed results, particularly with Hsp90 and eNOS.

## APPENDIX A

### A1. Streptozotocin induction protocol

REVISION DATE: 13/06/2012

#### **PURPOSE:**

To induce Type I diabetes in rats

#### **MATERIALS:**

Gloves

Lab Coat

Streptozotocin (STZ)

5X Stock Citric Acid/Citrate Buffer

- Anhydrous Citric Acid
- Sodium Citrate Dihydrate
- MilliQ Deionized Water

13M HCl

3 Falcon Tube

Sterile Filter

#### **EQUIPMENT:**

Biological Safety Cabinet

Weigh Scale

pH Meter

#### **PROCEDURE:**

*Preparing 5X Citric Acid/Citrate Buffer*

1. For a pH 4.6 buffer at 765 mM (5X stock solution), in a beaker, Add
  - i. 13.8g Anhydrous Citric Acid (Sigma) or 15.1g Citric Acid Monohydrate
  - ii. 23.8g Sodium Citrate Dihydrate (Sigma)Mix into
  - iii. 175mL of MilliQ water

The pH should be at 4.6, Add HCl or NaOH to adjust (do not over-shoot pH)

2. Once the proper pH is obtained, add MilliQ water until you are close to the 200 ml mark (pH will move slightly). If satisfied with the pH, adjust volume in a 250 ml graduated cylinder and filter in a 0.2µm filter.
3. Store at room temperature. This is your 5X stock solution.

*Making up Streptozotocin (STZ) for Injection*

**\*\*NOTE** Animals should be pre-weighed prior to making up STZ to ensure accurate amounts of STZ to be prepared.

1. Using pre-made buffer, put 1 mL of buffer in a 50 mL Falcon Tube and add 4 mL of distilled water filtered through a 0.2µm syringe filter. Check the pH. This gives you a working concentration of 153 mM
2. The desired pH is between 4.5-4.7. Under the fume hood, add 1 drop at a time of concentrated HCl to the buffer, checking pH in between until desired pH is reached.
3. Once pH is reached, add 1 mL distilled water (sterile filtered through a 0.2µm syringe filter as before). If pH is below 4.5, restart.
4. Weigh out an appropriate amount of STZ for the number of animals (see calculations below) that will be injected in a 15 minute time frame.

Ex. Rats will be injected at 20mg/kg, so for 10 animals at an ideal weight of 200g (avg. weight of rats to be injected), you will require a minimum of 40mg.

$$20\text{mg/kg} \times 0.2\text{kg} = 4\text{mg per animal}$$

The amount of STZ weighed out should be more than the minimum as some solution will be lost in filtering. (4mg (per animal) X 12 rats = 48mg total (0.048g)

5. Dissolve the STZ into buffer (keeping in mind a comfortable injection volume). Shake to dissolve powder (approx. 1min). Sterile filter using a 0.2µm syringe filter.  
Ex. 48mg STZ ÷ 3 mL buffer = 16mg/mL solution

$$4\text{mg} \div 16\text{mg/mL solution} = 0.25\text{mL}$$

6. STZ is time dependent and must be used within 5 minutes

#### *Injecting and Follow-Up of the Animals*

1. Promptly inject each rat with the solution (intraperitoneal) at a dosage rate of 20mg/mL (in this example, 0.25mL). Do not use anymore STZ solution more than 5 minutes after it has been dissolved in the sodium citrate buffer.
2. Dispose of any container having come into contact with the STZ (in either powder or dissolved form) into a biohazardous waste receptacle. Dispose of needles into a sharps container.
3. Return injected rats to their cage. Record the date of STZ injection and add a biohazard label to the cage (leave biohazard label on cage for at least 3 days following the last injection).
4. Repeat this procedure the following day.

5. Check blood glucose daily. Diabetes is achieved with two non-fasting blood glucose readings of  $>18$  mmol. Diabetes should be achieved after 5-8 injections (i.p. 20mg/kg).

*Reference:*

Low dose STZ induction protocol. Animal Models of Diabetic Complications Consortium AMDCC Protocols.2003

**A2. Subcutaneous insulin pellet protocol**

REVISION DATE: 13/06/2012

**MATERIALS:**

LinShin LinPlant Insulin Pellet  
Rat anesthetic - Isoflurane  
Ampicillin  
Sterile water  
1ml syringe with 25 g needle  
10% providone-iodine solution  
gauze (or swab)  
Tissue forceps  
Scalpel handle and blades (or scissors)  
Silk suture  
Needle drivers

**EQUIPMENT:**

Isofluorane Anaesthetic Machine  
Hair clippers  
Heat lamp

*Special Safety:*

Individual must wear lab coat and gloves before handling rodents. Any bite or scratch that breaks the skin must be thoroughly scrubbed with soap and water (report to Occupational Health and Safety, UWO).

**PROCEDURE:***Pellet implantation (for a rat):*

1. Anesthetize the animal using the isofluorane machine by placing it in the induction chamber. Set isoflurane to 4-5% with an O<sub>2</sub> flow rate of 1L/min. Open the stopcock valve so gas reaches the chamber. Keep in chamber until the animal is unconscious.
2. Remove the animal and place its nose in the nose cone, reduce the isofluorane to 3% to maintain the plane of anesthesia.
3. Shave the area where the pellet is to be implanted.
4. Using gauze (or a swab), apply 10% providone-iodine solution to the skin, followed by 70% ethanol, to disinfect the site of insertion.
5. Hold the skin with forceps and make a subcutaneous incision.

6. Cleanse a 12g trocar with 10% providone-iodine solution and insert it through the puncture site to a depth of at least 2 cm.
7. Using forceps, briefly immerse the pellet in 10% providone-iodine solution, rinse with saline and insert into the subcutaneous region.
8. Use 1 pellet for the first 350g of body weight.
9. Pinch the skin closed after the last pellet is inserted. Place a drop of 10 % providone-iodine solution over the opening.
10. Close the incision by suturing.
11. Place the animal under a heat lamp and monitor until it recovers from anesthesia.
12. Record on the cage card that insulin pellets have been implanted.

*Pellet removal:*

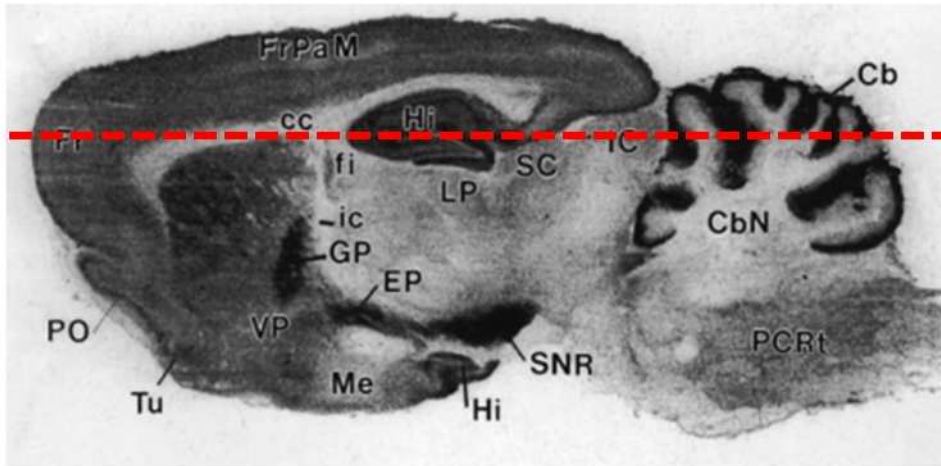
1. Anesthetize the animal as described above for implantation.
2. Shave and palpate the area of implantation to locate pellets. Sterilize this area by applying 10% providone-iodine solution followed by 70% ethanol.
3. Using a scalpel (or scissors), make an incision through the skin superficial to the location of the pellets.
4. Using forceps, remove the pellet. Some connective tissue may need to be cut away using scissors. Discard the pellet.
5. Close the incision by suturing.
6. Place the animal under a heat lamp and monitor until it recovers from anesthesia.
7. Record on the cage card that the pellets have been removed.

*References:*

<http://www.linshincanada.com>

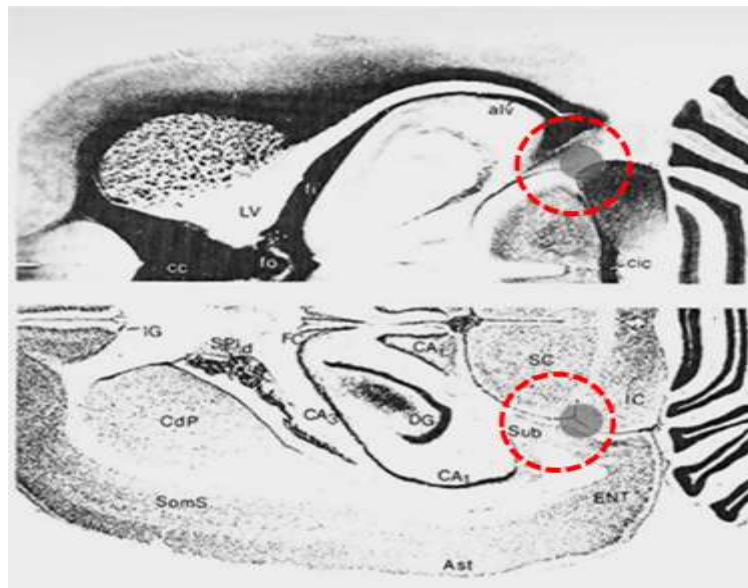


### A3. Brain Histology and Orientation



#### *A.3 Sagittal Section of the Rat Brain*

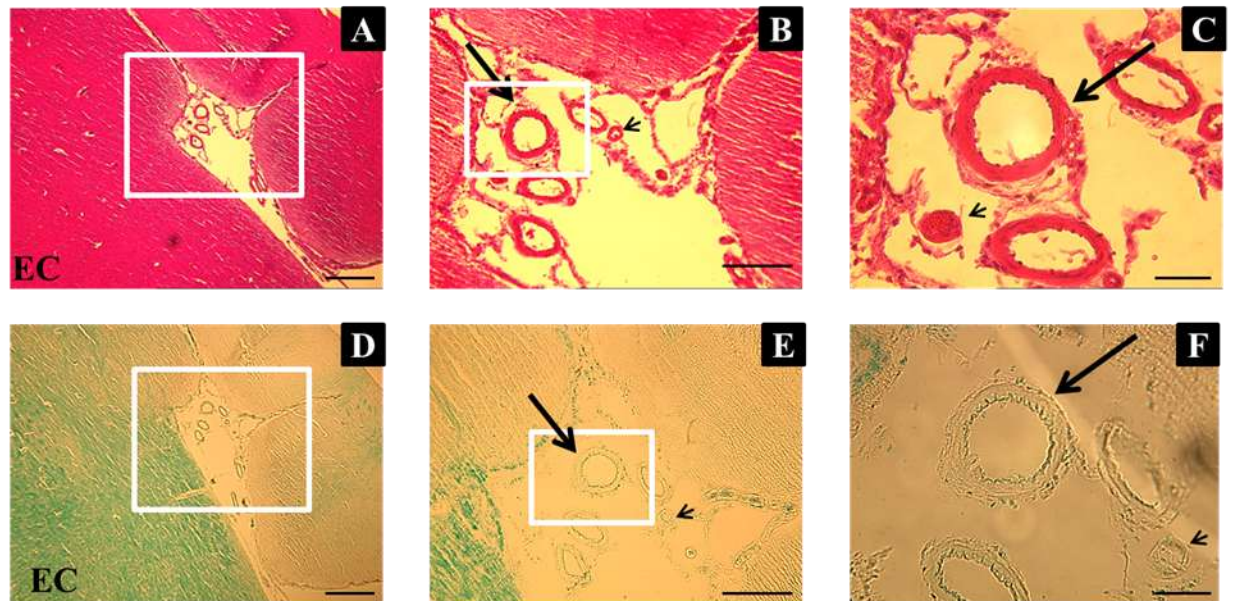
Dashed lines illustrate the approximate location of initial transverse sectioning of whole brains(17).



#### *A.3 Transverse Section of the Rat brain.*

The adapted image illustrates a prototype of the IHC sections. Red dashed circles enclose area indicating the area of cerebral blood vessel observation(19).

#### A4. Histochemical Stains

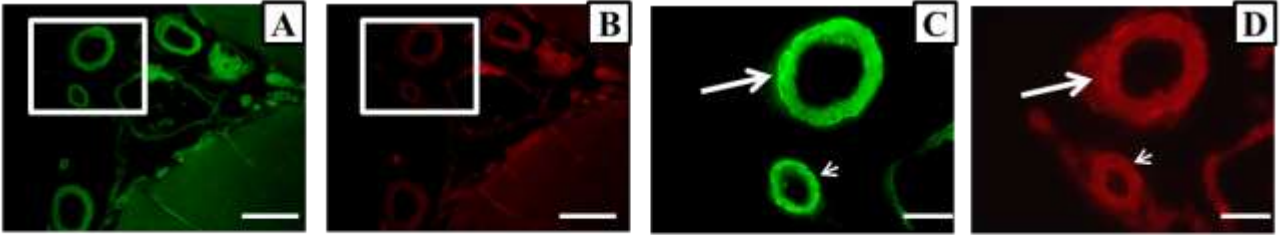


**Plate A4.** Histological Visualization of Cerebral Vessels in H & E (A-C) and LXFB (D-F) A,D(5X) boxed inset highlights the cluster of cerebral vessels adjacent to the entorhinal cortex (EC). B,E (16X) magnified inset from A,D. Large black arrows identify large cerebral vessels. Black arrowheads identify small cerebral vessels. C,F (40X) magnified inset from B,E.

Scale bar 5X=0.05mm    16X=0.5mm    40X=0.05mm

Histological Visualization of Cerebral Vessels in H & E and LXFB across a spectrum of magnification illustrates the anatomical region of cerebral vessels and delineates a small cerebral vessel from a large cerebral vessel. It further delineates cerebral matter from the adjacent vessels

### A5. Fluorescent Structural Comparison of Large versus Small Cerebral Vessels



**Plate A5.** Fluorescent Visual Comparison of Large and Small Cerebral vessels.

A-B (16X) boxed inset separated paired vessels from adjacent cluster and cortex.  
C-D (40X) comparatively distinguishes a large (white arrow) from a smaller vessel (white arrowhead).

Scale bar 16X=0.5mm

Scale bar 40X=0.05mm

For the purpose of the study, the vessels were separated into two sizes by their diameter and their morphological appearance. The larger vessels were identified as arterioles that encompassed 2-3 layers of smooth muscle cells and maintained a diameter range of about 0.135-0.14mm. The smaller vessels were identified as terminating arterioles with roughly a single layer of smooth muscle and maintained a diameter range of about 0.04-0.05mm.

## A6. Immunofluorescence Localization Protocol

REVISION DATE: 19/6/2013

**Proteins tested: Hsp70, Hsp90, iNOS, eNOS**

**Tissue tested: Paraffinized brain tissue:**

**Tissue Preparation:**

Whole brains were extracted 72 hours post-last exercise training bout for trained or following 10-week sedentary window.

Following which, brains were immediately placed in 4% paraformaldehyde renewed daily for 72 hours and placed in 70% ethanol until tissue processing.

Brain tissues were transversally split-sectioned and embedded in paraffin, 10µm transverse sections were mounted as pairs on frosted, poly-L-Lysine-coated, positively charged microscope slides and stored at room temperature. Each slide held two serial sections of the tissue in order to have negative controls.

**Immunofluorescent (IHC)**

**IHC Day 1**

1. Deparaffinize slides and antigen retrieved for approximately two hours with sodium citrate buffer [10mM sodium citrate, 0.05% tween-20, pH 6.0]
2. Transfer slides to coplin jars and wash in 0.01% Tween-20 in PBS (T-PBS) for 5 minutes, followed by two washes in phosphate buffered saline (PBS, 1M) for 5 minutes each.
3. Following the washes dry slides with Kimwipe and double circle each tissue section on the slide with a grease pen.
4. Block sections at room temperature with 10% goat serum in PBS for 1 hour in humidity chambers  
In the meantime, primary antibodies were thawed at room temperature and assembled as cocktail dilutions of in 1% Tween-PBS (TPBS) goat serum.
5. The primary antibodies were combined as follows:

Hsp70: Rabbit polyclonal anti-hsp72 (SPA-812 StressGen) + mouse monoclonal anti-alpha smooth muscle (ABCAM-18147).

Hsp90: Rabbit polyclonal to hsp90 (ABCAM-13495) + mouse monoclonal anti-alpha smooth muscle (ABCAM-18147).

iNOS: Rabbit polyclonal anti-iNOS (ABCAM-15323) + mouse monoclonal anti-alpha smooth muscle (ABCAM-18147).

eNOS: Rabbit polyclonal anti-eNOS/NOS Type III (BD Biosciences-610299) + mouse monoclonal anti-alpha smooth muscle (ABCAM-18147).

1° Antibody Cocktails	Antibody Identifier	1° Antibody Concentration
<b><i>Hsp70 + Smooth Actin</i></b>	Hsp70 (SPA-812)	1:100
	Smooth $\alpha$ actin (AB18147)	1:400
<b><i>Hsp90 + Smooth Actin</i></b>	Hsp90 (AB13485)	1:400
	Smooth $\alpha$ actin (AB18147)	1:400
<b><i>iNOS + Smooth Actin</i></b>	iNOS (AB 15323)	1:100
	Smooth $\alpha$ actin (AB18147)	1:400
<b><i>eNOS + Smooth Actin</i></b>	eNOS 1:100 (BD610299)	1:100
	Smooth $\alpha$ actin (AB18147)	1:400

- Following block, wash slides in PBS for three cycles of 5 minutes increment and dry with Kimwipes. One out of two possible tissue sections on every slide incubate with primary antibodies overnight in 4°Celsius. The other section was incubated with 1% TPBS goat serum in absence of primary antibodies in order to function as negative controls to test for non-specific staining.

### **IHC Day 2:**

- Wash slides in PBS for three cycles of 5 minute increment and dry with Kimwipes, following which incubate all sections secondary antibodies for 1 hour at room temperature.
- Make secondary Antibody Cocktail: Anti-mouse (1:400) + anti-rabbit secondary (1:400) antibodies in 1% goat serum in T-PBS

2° Antibody Cocktail Constituents	Antibody Identifier	2° Antibody Concentration
<b>Anti-mouse (Green fluorescence)</b>	Alexa Fluor®488 Goat Anti-Mouse IgG, Invitrogen,	1:400
<b>Anti-rabbit (Red fluorescence)</b>	Alexa Fluor®594 Goat Anti-Rabbit IgG, Invitrogen	1:400

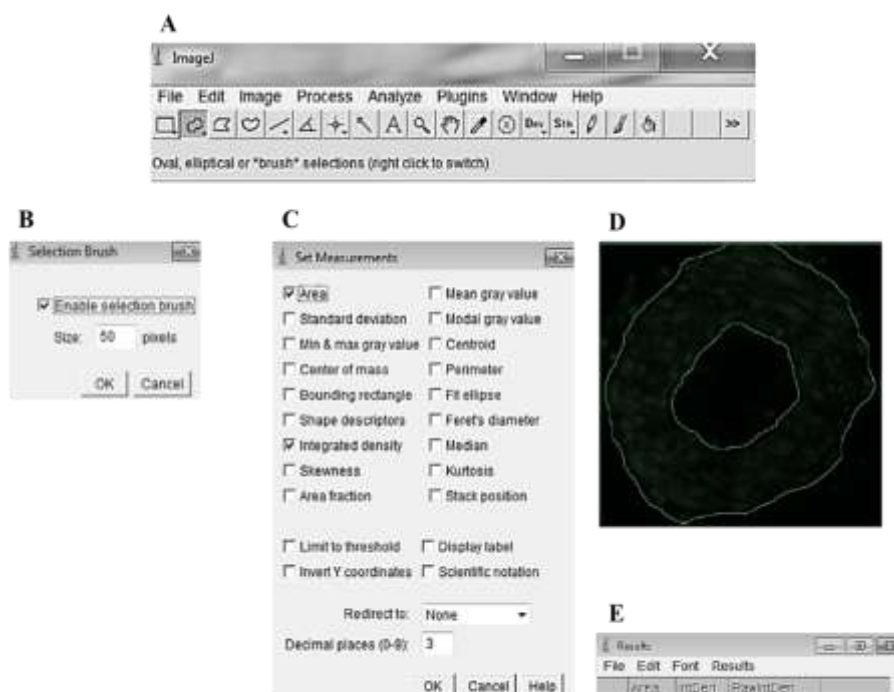
- Following secondary incubation, wash slides in PBS for three cycles (5 minute increments) and dry with Kimwipes.

10. For the purpose of fluorescent imaging, mount slides with a fluorescent mounting medium (Prolong® Gold Antifade Reagent containing DAPI, Invitrogen) containing 1% 4', 6-diamidino-2-phenylindole (DAPI), a fluorescent nuclear stain and then covered with glass cover slips (VWR® SuperSlips™ Micro Cover Glasses, Rectangular, No. 1, 24 X 55mm, CA48382-128). In order to maintain the fluorescent signal integrity and minimize photo bleaching, the store slides in a slide folder at 4°C until imaging.

## A7. Protein Abundance Acquisition

REVISION DATE: 19/06/2013

1. The fluorescent bright field microscopy carried out on (Zeiss Axiovert S100) and images were captured on Axiovert S100 at 16X and 40X magnification, using Northern Eclipse 6.0 (Empix Imaging Inc) software.
2. The protein content/abundance signal for Hsp70, Hsp90, iNOS and eNOS were extracted as using Image J. Specifically, protein content was determined by outlining the blood vessel of interest (D) and calculating the integrated pixel density (signal) using the selection brush (B), on Image J and the same technique was used to determine the integrated pixel density for the negative control of the same blood vessel.
3. To obtain the relative protein density, the pixel density of the negative control was subtracted from the pixel density of the experimental sample. The difference in pixel density provided values that were used to indicate fluorescence signal intensity and thus quantity of protein. For the collection of data for small and large vessels, this mode was repeated for four blood vessels from the same animal and averaged to give a signal protein value for the particular animal.



**A5. Image J Screen shots**

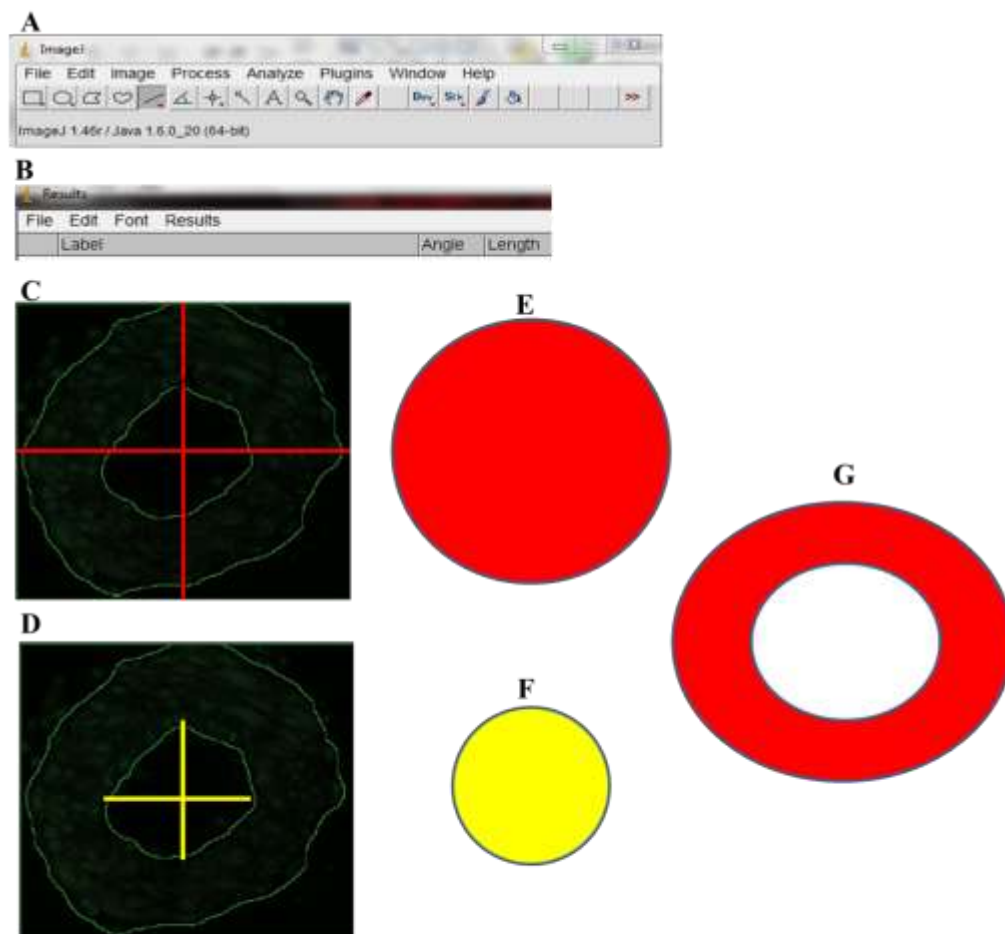
A-E are relatively sequential for signal density (integrated density) quantification.



### A8. Smooth Muscle Area Calculations

For comparative analysis between vessel size and protein content, smooth muscle area (SMA) was calculated for each vessel where the protein content was calculated. In addition, SMA calculations allowed for protein content adjusted to the area. The measures were collected from Image J. (A-B)

For SMA calculations, two full length perpendicular diameters from the outer perimeter of each vessel (C) were averaged to obtain a diameter for the outer circle. Likewise, two perpendicular diameters from the inner 'endothelial' perimeter of each vessel were averaged to obtain a diameter for the inner circle (D). Both the diameters were implemented to manually calculate the respective circular areas (E, F). The smaller circle area (F) was then subtracted from the larger (E) circle in order to obtain the SMA for that specific vessel (G).



#### *A6. Schematic Diagram of SMA Calculations*

A-G are relatively sequential for calculation of final SMA.



## APPENDIX B

### B.1 Ethics Approval

#### THE UNIVERSITY OF WESTERN ONTARIO – ANIMAL USE SUBCOMMITTEE PROTOCOL RENEWAL FORM

Protocol # 2008-095 Noble

##### A. PROJECT/INVESTIGATOR INFORMATION

Investigator Name: Earl Noble

Current Protocol # 2008-095

Project Title: *This is a NEW title* - Yes ☐

Innovation to reduce cardiovascular complications of diabetes at the intersection of discovery, prevention and knowledge exchange

##### B. INVESTIGATOR DECLARATION

I. All animals used in this research project will be cared for in accordance with the recommendations of the Canadian Council on Animal Care and the requirements of the provincial legislation entitled, "The Animals for Research Act," of the Province of Ontario.

II. I confirm that this *Animal Use Protocol* accurately represents the proposed animal use.

III. I accept responsibility for procedures performed on animals in this project.

IV. I will ensure that any individual who will perform any animal-related procedure(s) within this protocol will complete all related mandatory training AND will be made familiar with the contents of this document.

1. I support the above declaration - YES ☒ Today's Date *mm/dd/yy*: October 30/09

2. By checking 'YES' in this section, I authorize the submission of this form and its electronic delivery to [auspc@uwo.ca](mailto:auspc@uwo.ca)  
YES ☒ NO ☐

AUS APPROVAL - AUS Office Use Only -

Veterinary Authorization by Click Here  
Authorization Date (*mm/dd/yy*) / /

Signature:

##### C. CHANGES AT RENEWAL CONFIRMATION – Pick One Only

☒ I confirm that NO CHANGES and/or NEW ELEMENTS to this AUP - other than staffing - have occurred since the last AUS Form submission.

☐ I confirm that all CHANGES and/or NEW ELEMENTS to this protocol not previously submitted WILL BE sent via a *Protocol Modification* form along with this *Protocol Renewal Form*.

# THE UNIVERSITY OF WESTERN ONTARIO – ANIMAL USE SUBCOMMITTEE PROTOCOL RENEWAL FORM

Protocol # 2008-095 Noble

## D. THREE R's PROGRESS REPORT

Describe any progress made with respect to the Three R's of replacement, reduction and refinement of animal use during the PAST PROTOCOL YEAR. See the CCAC 3 R's Microsite: <http://www.ccac.ca/en/alternatives/>

We have used less animals than anticipated in the past year because we have made more use of the tissues generated by the animals that we employed.

- ☒ No additional animals were used during the past Protocol Year.  
☒ No additional animals were requested during the past Protocol Year.

## E. ANIMAL USE COMPLICATIONS PROGRESS REPORT

Describe any complications beyond the expected experimental outcomes and end points encountered relative to animal use – e.g. unpredicted outcomes and any unexpected animal pain, distress, or mortality - AND indicate measures being undertaken to resolve these complications for the PAST PROTOCOL YEAR.

- ☒ No complications were encountered relative to animal use during the past Protocol Year.

## F. ANALGESIA ADMINISTRATION UPDATE

Choose One of the Following Statements:

1. Analgesics were administered as outlined within the *Animal Use Protocol* ☐ \*If checked, please list all analgesics currently used below:

2. Analgesics are not indicated in this *Animal Use Protocol* ☒

## G. PROTOCOL PERSONNEL UPDATE

Complete for ALL Staff Working Under This Protocol for This Renewal Year

### TRAINING INFORMATION

**CCAC Mandated Training Requirements** – All personnel working with live animals require CCAC mandated training including the 'Basic Animal Care & Use Web-CT Course' and related hands-on 'Workshops'. Completion of the 'Basic Animal Care & Use Web-CT Course' once every 5 years is mandatory for ALL personnel, including the Principal Investigator. The Animal Use Subcommittee will be informed of all personnel with incomplete training beyond 1 month of notice.

**Workshop Enrolment Detail** – The 'Workshop' requirements are determined by the species and procedures associated with each individual



# THE UNIVERSITY OF WESTERN ONTARIO – ANIMAL USE SUBCOMMITTEE PROTOCOL RENEWAL FORM

Protocol # 2008-095 Noble

listed below. All personnel listed below will be contacted directly via the email address listed below for auto-enrolment in all 'Workshop' requirements. Previous hands-on 'Workshops' attended at another research institution may be accepted; please submit training documentation with this form. For additional training requirement detail and associated costs, go to [http://www.uwo.ca/animalwebsite/SS/Content/Teaching and Courses.htm](http://www.uwo.ca/animalwebsite/SS/Content/Teaching%20and%20Courses.htm)

COMPLETE ALL COLUMNS BELOW PER PERSON				If 'YES' to HANDS-ON ANIMAL WORK, Complete This Section PER SPECIES											
FIRST NAME <small>Repeat For Each Species Used</small>	LAST NAME <small>Repeat For Each Species Used</small>	ROLE <small>(Role within this Protocol)</small> Researcher Staff Student	EMAIL <small>*Mandatory Field*</small> UWO & Affiliated Institution Emails Preferred	HANDS ON ANIMAL WORK? YES or NO	SPECIES <small>One Species Per Row Use an Additional Row for Each Species</small>	Expected START DATE <small>mm/dd/yy</small>	PROCEDURES PER SPECIES  1=Basic Handling 2=Health Monitoring 3=Blood Collection 4=Injections 5=Anaesthesia 6=Surgery-Recovery 7=Surgery-Non-Recovery 8=Euthanasia/Post Mortem 9=Other, Provide Detail Below, e.g. suturing								
Earl	Noble	Researcher	enoble@uwo.ca	Yes	Rat	1/01/09	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
Tomasz	Dzialoszynski	Staff	tdzialos@uwo.ca	Yes	Rat	11/01/09	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
Oscar	Campos	Student	ocamp@uwo.ca	Yes	Rat	11/01/09	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
Jordan	Silver	Student	jsilver@uwo.ca	Yes	Rat	11/01/09	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
Mehrbod	Estaki	Student	mesaki@uwo.ca	Yes	Rat	11/01/09	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
PROCEDURE #9 DETAIL sutures after implantation of insulin pellets is necessary (EN and TD only); gavage for ginseng															
EMERGENCY AFTER HOURS CONTACT NAMES & NUMBERS - NO LAB PHONE NUMBERS -						PRIMARY EMERGENCY CONTACT LAST NAME & INITIAL : Noble E									
If applicable, provide POST-OP CARE personnel detail						SECONDARY EMERGENCY CONTACT LAST NAME & INITIAL : Dzialoszynski T									
If applicable, provide MONITORING personnel detail						Emergency #: 519-472-8399 Lab #: 88373 Email: enoble@uwo.ca									

## CURRICULUM VITAE

**Adwitia Dey**

### EDUCATION:

**University of Western Ontario**, MSc Candidate-Kinesiology-Integrated Physiology (2011-2013)  
**University of Toronto**, Hons B.Sc., Human Biology & Animal Physiology (2011)

### AWARDS:

Pennsylvania State University Graduate Graham PhD Fellowship Award-Physiology (2013-2015)  
 Western Kinesiology Graduate Board Student of the Month (May 2012)  
 Western Graduate Research Scholarship (WGRS; 2011-2013)

### RESEARCH EXPERIENCE:

**Exercise Biochemistry & Molecular Biology Laboratory: Research Assistant**

Advisors: Dr.Earl G.Noble, Dr.C.W.J Melling, PhD, & Dr.J.Murias, PhD 9/2011-8/2013  
 Western University, Kinesiology

**Neurophysiology Laboratory: Research Assistant**

Advisor: Dr.Melanie A.Woodin, PhD 9/2010-3/2011  
 University of Toronto, Cells & Systems Biology/Neuroscience

**Flow Cytometry Research Laboratory: Immunopathology Student**

Advisor: Dr. Frederic Preffer, PhD 5/2010-8/2010  
 Harvard University Medical School, Pathology

**Immunohistochemistry & Anti-GBM Diagnostics Laboratory: Immunopathology Student**

Advisor: Bernard Collins 5/2010-8/2010  
 Massachusetts General Hospital, Pathology

**Limnology Laboratory: Work Study Student**

Advisor: Dr. Helene Cyr, PhD 9/2009-3/2010  
 University of Toronto, Ecology and Evolutionary Biology

**Molecular Plant Virology Laboratory: Undergraduate Volunteer**

Advisor: Dr.Mounir Abou-Haider, PhD 9/2008-6/2009  
 University of Toronto, Cells & Systems Biology

**Neurophysiology Laboratory: Research Opportunity Program (ROP) Course**

Advisor: Dr.Zhong-Ping Feng, PhD, M.D 9/2006-9/2007  
 University of Toronto, Faculty of Medicine- Physiology/Neuroscience

**Extracellular Matrix Engineering Research Laboratory: Summer Student**

Advisor: Dr.Jeffery Ruberti, PhD 6/2005-8/2005  
 Northeastern University, Egan Research Center-Mechanical Engineering

**Massachusetts General Hospital Flow Cytometry Diagnostics Core: Summer Student**

Advisor: Dr. Frederic Preffer, PhD 6/2004-8/2004  
 Massachusetts General Hospital Cancer Care Center

**Massachusetts General Hospital Stem-Cell Laboratory: Summer Student Volunteer**

Advisor: Martin L. Caron, MS MT 7/2003  
 Massachusetts General Hospital, Bone Marrow Processing Laboratory

## PUBLICATIONS:

Murias JM, **Dey A**, Campos OA, Estaki M, Hall KE, et al. (2013) High-intensity endurance training results in faster vessel-specific rate of vasorelaxation in type 1 diabetic rats. PLoS ONE 8(3): e59678. doi:10.1371/journal.pone.0059678.Mar.2013

T.D.Olver, K.N.Grisé, M.W. McDonald, **A.Dey**, M.D Allen, J.C.Lacefield, E.G. Noble, J.K.Shoemaker. The relationship between mean arterial pressure and peripheral nerve blood flow in insulin-treated type 1 diabetic rats. (Submitted to: Cerebral Blood Flow & Metabolism JCBFM-0315-13-ORIG)

Grisé, K. N., Olver, T. D., McDonald, M. W., **Dey, A.**, Lacefield, J.C., Shoemaker, J.K., A., Noble, E. G., Melling, C. W. J. The effects of exercise training on indices of cardiovascular autonomic neuropathy in STZ-induced type 1 diabetic rats treated with insulin. (Near submission to Journal of Applied Physiology-Sept2013)

C.W.J Melling, M.W. McDonald, K.E. Hall, M.Jiang, **A.Dey**, E.G.Noble. Glycemic responses and protection from ischemia-reperfusion injury in insulin-treated T1DM rodents using various modalities of exercise training. (Near submission to Journal of Applied Physiology-Sept2013)

T.D.Olver, M.W. McDonald<sup>2</sup>, K.N. Grisé, **A.Dey**, M.D. Allen, P.J. Medeiros, J.C. Lacefield ,D.N. Jackson, C.L. Rice, C.W.J Melling, E.G. Noble, J.K Shoemaker. *Exercise is medicine: exercise training preserves insulin-stimulated nerve vasodilation in rats with insulin-treated experimental diabetes.*(Near submission to Journal of Applied Physiology-Sept 2013)

## PUBLISHED ABSTRACTS

1. **A.Dey**, MW.Mcdonald, K.Hall, EG.Noble, CWJ.Melling,*The Effect of Exercise Modality on the Ventricular Myocardial HSP70 Expression in a Type 1 Diabetic Rat Model.* Diabetes Supplement Vol.62 July#2013-A-4682

2. Murias JM, **Dey A**, Campos OA, Estaki M, Hall KE, Melling CWJ, Noble EG. *Vessel-specific Vasorelaxation, e-NOS Protein Content and Lumen-to-wall Ratio in Rats.* Med Sci Sports Exerc; Volume 45(5) Supplement May 2013 p S2727

3. MW.McDonald, **A.Dey**, KN.Grise, TD.Olver, JK.Shoemaker, EG. Noble, CWJ Melling. *The influence of training on exercise-induced hypoglycemia in Type 1 diabetes mellitus rats.* Diabetes Supplement Vol 62 July #2013-A-4524)

4. TD Olver, KN Grise, MW McDonald, **A Dey**, AW Collins,EG Noble,CWJ Melling, K Shoemaker. *Blood Pressure and Vascular Function in Sprague-Dawley Rats With Diabetes.* Diabetes Supplement Vol 62. July #2013-5-LB

5. TD Olver, KN Grise, MM McDonald, **A Dey**, EG Noble, CWJ Melling, JC Lacefield, JK Shoemaker. *The effect of chronic low grade hyperglycemia on basal nerve blood flow values in Sprague-Dawley rats.* FASEB Journal 2013.27:901.5

6 TJ Hazell, TD Olver, H Kowalchuk, MW McDonald, **A Dey**, KN Grise, P Lavery, HA Weiler. Endurance exercise does not protect the decrease in bone associated with Type 1 diabetes in adult rats. FASEB Journal. 2013.27:712.23

7. T.D.Olver, M.W.McDonald, K.N.Grise, **A.Dey**, E.G.Noble, C.W.J.Melling, J.C.Lacefield, J.K.Shoemaker. Exercise training mitigates the loss of insulin-stimulated sciatic nerve arterial vasodilation associated with chronic low-grade hyperglycemia.. Applied Physiology, Nutrition, and Metabolism, 2012, 37(S1): S27, 10.1139/h2012-104

## ACADEMIC PROCEEDINGS/PRESENTATIONS

**A.Dey**, J.M. Murias, C.W.J. Melling, E.G. Noble: *Exercise modality mediated vessel-specific rate of vasorelaxation and the vascular e-NOS expression in Type 1 Diabetic Rats*. Western Health Science Research Forum. Mar.2013.

**A.Dey**, M.W.McDonald, K.Hall, M.Jiang, E.G.Noble & C.W.J.Melling: *The effect of Aerobic & Resistance Exercise training on HSP70 Expression in Type 1 Diabetic Rat Myocardium*. Schulich Medicine & Lawson Health Institute Diabetes Research Day. Nov.2012

M.W. McDonald, K.N.Grise, T.D.Olver, **A.Dey**, J.K.Shoemaker, E.G.Noble, C.W.J. Melling. *The Effect of Consecutive Days of Aerobic Exercise on Exercise-induced Hypoglycemia in Type 1 Diabetic Rats*. Schulich Medicine & Lawson Health Institute Diabetes Research Day. Nov.2012

T.D.Olver, M.McDonald, **A.Dey**, E.G.Noble, C.W.J. Melling, J.D. Lacefield, J. K.Shoemaker. *Using a Physiological Model of Type I Diabetes to Investigate the Effect of Low Grade Hyperglycemia on the Peripheral Nerve Arterial Autoregulation Index*. Schulich Medicine & Lawson Health Institute Diabetes Research Day. Nov.2012

**A.Dey** & Z-P.Feng. *Role of Calcium Binding Proteins on the Lymnaea stagnalis memory formation and retention*. University of Toronto Faculty of Arts and Science Research Opportunity Program (ROP) Poster Day. Apr.2007

## TEACHING EXPERIENCE

- |  |                |
|--|----------------|
| 1. Western University-Secondary Course Instructor, Teaching Assistant<br><i>KIN 1088: Introduction to Sports Psychology- Professor R.LaRose.</i> | 9/2011-12/2012 |
| 2. Western University-Teaching Assistant<br><i>KIN 1080: Introduction to Psychomotor Behavior- Dr.Matthew Heath.</i>                             | 1/2012-4/2013  |

## PROFESSIONAL ADMINISTRATIVE EXPERIENCE

- |  |               |
|--|---------------|
| 1. UWO-Society of Graduate Students (SOGS) Alternate Councilor               | 4/2012-4/2013 |
| 2. UWO-Western Serves-Re-Forest London Volunteer                             | 9/2011-8/2013 |
| 3. U of Toronto-Think First BRAIN Day Association, Neuroscience Sr.Volunteer | 9/2008-8/2013 |
| 4. U of Toronto-Cooks, President   | 9/2006-8/2007 |